1973

Studies on the Mode of Action of Benomyl in Neurospora Crassa.

Mildred Kathleen Borck
Louisiana State University and Agricultural & Mechanical College

STUDIES ON THE MODE OF ACTION OF BENOMYL IN *NEUROSPORA CRASSA*

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Microbiology

by
Mildred Kathleen Borck
B.S., University of Florida, 1964
December, 1973

ACKNOWLEDGMENT

I wish to express my sincere appreciation to Dr. H. D. Braymer, who provided guidance and reassurance throughout the course of this study and who willingly gave of his time for lengthy discussions of ideas. I am also endebted to Dr. A. D. Larson for his generous loan of equipment and supplies and to all the members of my committee for their critical advice. Dr. S. Chang and Dr. N. R. Miller kindly offered assistance and recommendations relative to the nucleic acid aspects of this investigation. I am most deeply grateful for the competent technical aid of Miss Pamela Abraham, who has donated cheerful and untiring assistance during the course of this research. Thanks are extended to my fellow graduate students, especially Marvin Fleenor, for their helpful suggestions. In addition, I would like to thank Mrs. Gayle LaPiccolo for aid with the electron microscopy, Mrs. Janie P. Stilling for the typing of this manuscript and Miss Lynn Howington for preparation of the figures.

I wish to express special gratitude to my husband, Jim, whose patience, understanding and encouragement have been invaluable. Lastly, I thank my parents, Dr. and Mrs. D. O. Spinks, for their unfailing support; it was they who instilled in me a love for research.

TABLE OF CONTENTS

LIST OF TABLES

TABLE Page

APPENDIX TABLE

LIST OF FIGURES

FIGURE Page

ABSTRACT

Growth of wild-type Neurospora crassa was completely inhibited on solid medium by 0.25 μg/ml benomyl [methyl-1-(n-butylcarbamoyl)-2-benzimidazole carbamate]. In liquid medium, concentrations of benomyl up to 1.5 μg/ml did not prevent germination of N. crassa conidia; however, hyphal elongation was suppressed and there was some increase in dry weight of treated cultures up to 16 hours after addition of benomyl. Cytological studies revealed that treated mycelia exhibited gross morphological distortions with a granular appearance of the cytoplasm. Electron microscopy of benomyl treated cells showed no aberrations of mitochondria, nuclear or plasma membrane or endoplasmic reticulum, but some increase in the amount of vacuolation was observed and cell walls were 2-3 times as thick as cell walls of normal hyphae. Cell wall analyses demonstrated that cell wall synthesis continued in the presence of benomyl and that resultant cell wall material differed only slightly in composition, that difference being in the ratio of glucosamine to galactosamine.

Benomyl (1 μg/ml) did not affect protein synthesis during a 16-hour period after addition of the fungicide. Determination of RNA and DNA content of benomyl-grown cells indicated that the effects of benomyl on nucleic acid synthesis are delayed rather than immediate. Inhibition of

RNA synthesis by benomyl (1 μg/ml) was 10-20% by 4 hours and 81% by 16 hours after treatment. DNA synthesis was reduced 25% at 4 hours and was almost totally inhibited by 16 hours. Incorporation experiments (in vivo) with labeled nucleotide precursors did not show inhibition of nucleic acid synthesis up to 2 hours after addition of benomyl, demonstrating that the toxicant did not have a direct effect on incorporation of nucleotides into polynucleotides. Measurement of the pool sizes of bases, nucleosides and nucleotides have indicated that benomyl causes a reduction of AMP and ADP, a redistribution in amounts of UMP, UDP and UTP and an accumulation of UDP-glucose. These results imply that benomyl interferes with the synthesis of nucleotides, particularly purine nucleotides, so as to deplete the supply available for nucleic acid biosynthesis. The ramifications of these effects are then reflected in the formation of more cell wall material and in the alterations in morphogenesis which have been observed.

Studies on ascospore segregation from sexual crosses of Neurospora indicated there was no effect of benomyl on meiosis or mitosis. Filtration enrichment experiments demonstrated that benomyl had only very weak, if any, mutagenic properties.

Benomyl-resistant mutants were selected following ultraviolet irradiation of wild-type Neurospora conidia. Genetic mapping of 15 resistant mutants revealed that tolerance to benomyl is conferred by a single gene located

2.9 map units from *ylo-1*, 32.1 units from *chol-2* and 17.9 units from *tryp-2* in linkage group 6 of *Neurospora*. Mutants had variable resistance levels, and some were found to grow in the presence of greater than 15 μg/ml benomyl.

INTRODUCTION

Man has been concerned since ancient times with the protection of his crops against infection by phytopathogenic fungi; yet before the introduction of Bordeaux mixture by Millardet in 1881 (Crowdy, 1970), sulfur was the only fungicide used to protect fruit trees and vegetables. Since 1934, when Tisdale and Williams patented the first organic fungicide, a dithiocarbamate (McCallen, 1969), a tremendous number of organic compounds possessing antifungal activity have been synthesized or isolated from natural sources.

In the last decade many of these organic fungicides have been considered most promising because of their systemic properties. First, application may be restricted to infected plants only; secondly, the mode of action is directed against the pathogen at the site of infection. The systemic fungicides also circumvent problems caused by imperfect coverage of sprays, dilution by rainfall and inadequate coverage of new growth. The desirable attributes which a systemic compound does possess are obvious: efficient translocation, resistance to degradation or detoxification, and specific interference with the vital processes of the pathogen.

In the 1960's several benzimidazole derivatives were introduced as systemic fungicides; these derivatives were attractive to scientists because they possessed wide-spectrum

antifungal activity and low mammalian toxicity. More recently, these compounds have become increasingly important for contemporary agricultural practices, and they have stimulated scientific interest in attempting to determine the mechanisms of action exhibited by these compounds. Benomyl, methyl-1-(n-butylcarbamoyl)-benzimidazolecarbamate, is one of the benzimidazole derivatives which has shown high toxicity against Ascomycetes and some Basidiomycetes. Thus, this investigation examines benomyl's mode of action in a sensitive Ascomycete, Neurospora crassa. The cytological and biochemical effects of benomyl have been observed in an effort to establish the primary site of inhibition. In order to further clarify the fungicide-fungus interactions, this project includes a study of the genetics and characteristics of benomyl-resistant mutants.

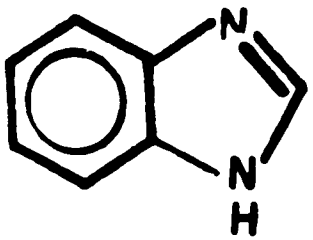
LITERATURE REVIEW

As early as 1951, Horsfall and Rich studied heterocyclic nitrogen compounds for efficacy as fungicides by testing their effects on spore germination (Frear, 1955). Included in the group were derivatives of indole, imidazole, pyrimidines, acridines and numerous others. The conclusions drawn from this study were as follows: 1) unsubstituted heterocyclic nitrogen compounds were not very toxic to fungi, 2) alkyl or aryl substitutions attached to the heterocyclic structures increased their fungitoxicity, 3) polar groups, such as $-NH_2$, $-NO_2$ and -OH, attached to the heterocyclic ring increased toxicity, and 4) a change in valence of a ring nitrogen from 3 to 5 by producing quaternary ammonium compounds greatly increased antifungal activity (Frear, 1955).

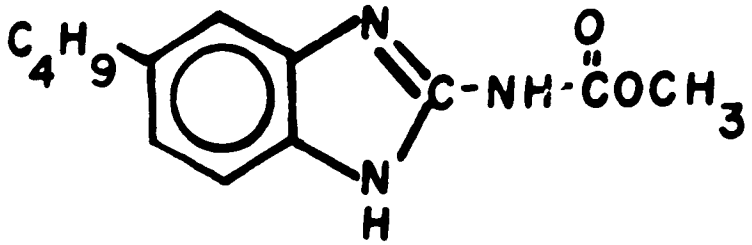
Although benzimidazole and its derivatives were not included in this report, the research which has been done on fungitoxicity of benzimidazoles indicates that these conclusions are generally applicable, as will become evident in the succeeding discussion of those benzimidazole compounds which have shown promise as fungicides. Structures of benzimidazole and derivatives are revealed in Figure 1.

Benzimidazole (1,3-benzodiazole). Unsubstituted benzimidazole has been found inactive against fungi (Fuchs et al., 1970; Edgington et al., 1971). Apparently, the addition of substituents on the ring moiety is important for fungal

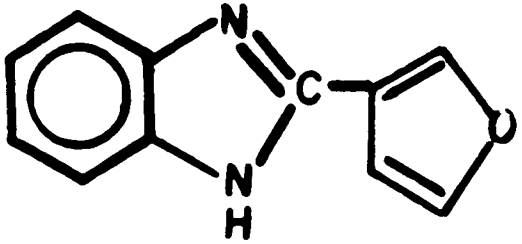
Figure 1. Structures of benzimidazole and the derivatives which have shown antifungal activity.



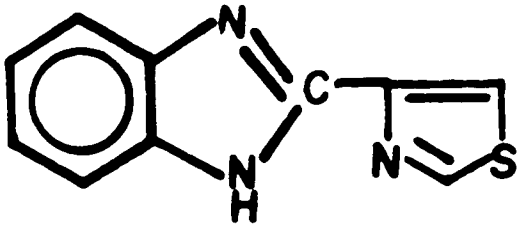
BENZIMIDAZOLE



PARBENDAZOLE



FURIDAZOLE



THIABENDAZOLE

toxicity. Benzimidazole does inhibit growth of yeasts and bacteria (Woolley, 1944) and the replication of some viruses (Hitchings and Elion, 1963; Stutzenberger and Parle, 1972); however, the effect on viral reproduction may well be the result of interference with vital processes in the host cell, i.e., nucleic acid or protein synthesis or respiration.

Furidazole [2-2-(furyl)-benzimidazole, Bay 33172]. Edgington et al. (1971) reported that furidazole displayed a pattern of selective toxicity to certain groups of Deuteromycetes and Basidiomycetes identical with that of two other benzimidazoles, thiabendazole [2-(4'-thiazolyl)-benzimidazole] and benomyl. Such group selectivity may indicate that all three compounds have a common mechanism of action. In general, furidazole has proven less toxic to fungi than other derivatives (Bartels-Schooley and MacNeill, 1970) suggesting that the substituent attached to the imidazole portion of the molecule also plays a role in toxicity.

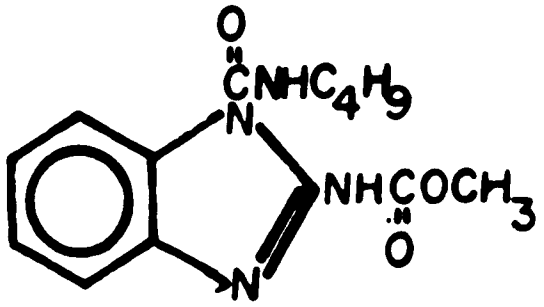
Parbendazole [5(6)-n-butyl benzimidazolecarbamate]. Parbendazole has not been widely used as a systemic fungicide and Maxwell and Brody (1971) have shown it to be the least active of a group of benzimidazole derivatives tested on a variety of plant and animal pathogenic fungi. Addition of a butyl moiety narrowed the spectrum of toxicity thus increasing selectivity.

Thiabendazole [2-(4'-thiazolyl)-benzimidazole, Merck TBZ]. Thiabendazole was first introduced in 1961 as an anthelminthic (Brown et al., 1961). Later, Robinson and coworkers (1964)

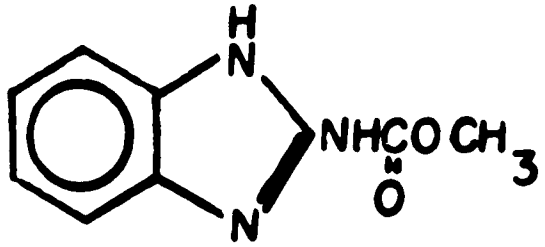
reported that thiabendazole acted as a fungicide against certain pathogenic dermatophytes at concentrations as low as 1-8 μg/ml of medium and was fungistatic to several saprophytes at 1-20 μg/ml. Since the discovery that thiabendazole was systemic in sugar beets and fungitoxic against Cercospora (Erwin, 1969), it has gained recognition as an effective broad spectrum systemic agent (Daines and Snee, 1969; Hine et al., 1969; Solel, 1970; Edgington et al., 1971; Maxwell and Brody, 1971). According to available studies, thiabendazole is fungistatic, rather than fungicidal, to plant pathogens. It is not as effective as benomyl, but more so than either furidazole or parbendazole (Hine et al., 1969; Fuchs et al., 1970; Bartels-Schooley and MacNeill, 1971; Maxwell and Brody, 1971). In addition to its antifungal properties, thiabendazole is also active against some bacteria, yeasts and Actinomycetes (Edgington et al., 1971).

Benomyl [methyl-1-(n-butylcarbamoyl)-2-benzimidazolecarbamate, Benlate, duPont 1991]. In 1967 duPont de Nemours and Co. introduced a new compound, benomyl (Figure 2), which acts as a nematocide (Miller, 1969), mite ovicide (Delp and Klopping, 1968) and a broad spectrum fungicide having a unique combination of protective, curative (Daines and Snee, 1969) and systemic properties. It is more potent than any of the benzimidazoles tested, and it has elicited considerable interest since the first reports by Delp and Klopping (1968). Benomyl exhibits almost complete lack of phytotoxicity (Fuchs et al., 1970) and resistant mutants have

Figure 2. Structures of benomyl and MBC.



BENOMYL



MBC

been discovered in only a few pathogens (Schroeder and Providenti, 1969; Bollen and Scholten, 1971). Most research groups (Hine et al., 1969; Bollen and Fuchs, 1970; Clemons and Sisler, 1971; Maxwell and Brody, 1971) have observed that benomyl acts as a fungistat rather than a fungicide.

Using fungi in all taxonomic groups, Bollen and Fuchs (1970) and Edgington et al. (1971) established that benomyl is effective against Ascomycetes and some Deuteromycetes and Basidiomycetes. Because of its existing group specificity, these authors proposed that there must be some correlation between benomyl sensitivity and morphogenesis of conidia.

Clemons and Sisler (1969) and Kilgore and White (1970) demonstrated that benomyl is rapidly hydrolyzed in aqueous solution to methyl-2-benzimidazolecarbamate (MBC, Figure 2). Fuchs et al. (1972) showed, by spectral analysis of benomyl in aqueous solution, that benomyl was converted to MBC in approximately 45 minutes at pH 4-8 and 25 C; this conversion was accelerated by alkaline pH and heat. Boiling or autoclaving solutions caused extensive reduction in fungistatic activity; the loss was ascribed to conversion of MBC to a non-toxic compound such as aminobenzimidazole.

Due to the rapid hydrolysis of benomyl to the more stable methyl ester, it has been suggested that MBC is primarily responsible for the fungitoxicity of benomyl. This theory seems credible in view of several observations. First, studies carried out on plants treated with benomyl revealed that MBC is rapidly produced and systemically

transported into the foliage (Erwin, 1969; Sims et al., 1969; Peterson and Edgington, 1970, 1971; Fuchs et al., 1972). Chromatography of plant extracts showed a predominance of MBC with only trace amounts of benomyl remaining. Secondly, comparison of the toxicity levels of benomyl and MBC (Maxwell and Brody, 1971) demonstrated that the two compounds had the same minimal inhibitory concentration values (the concentration in which the test organism would no longer grow) for 10 out of 11 selected fungi. Clemons and Sisler (1969) provided additional support by finding toxicity levels of benomyl and MBC toward N. crassa and Rhizoctonia solani were equal. However, the effect of benomyl on Saccharomyces pastorianus was 30 times that of MBC. The authors therefore proposed the mode of action of the two compounds was identical in fungi, but not in yeasts.

Mode of Action

The characterization of the mode of action of a fungicide must include a discussion of the interactions between the fungus and its inhibitor. There are many vital processes continually functioning within a growing organism, and all of them are interrelated. The target of inhibition may involve any one or more of these processes, thus it seems relevant to consider the action of a toxicant upon the various pathways of metabolism. The following discussion will review what is presently known about the cytological,

biochemical and genetic effects of benzimidazole compounds on cells.

Effects on Germination and Growth

Several researchers have reported that benzimidazole has no effect on germination or growth of fungi (Fuchs et al., 1970; Edgington et al., 1971; Stutzenberger and Parle, 1972). Yet Woolley (1944) discovered that growth of yeasts (Saccharomyces cerevisiae and Endomyces vernalis) and bacteria (Escherichia coli and Streptococcus lactis) was suppressed by benzimidazole. The concentrations necessary for inhibition, however, were quite high when compared to the activity of benzimidazole derivatives against fungi.

Gottlieb and Kumar (1970) studied the reaction of Penicillium atrovenetum and Aspergillus oryzae to various concentrations of thiabendazole. In both organisms mycelial elongation was restricted and hyphae became malformed when as little as 2 μg/ml thiabendazole was added to pregerminated cultures. Much higher levels (10-40 μg/ml) were required to inhibit germination of both fungi. Solel (1970) has noted similar effects on growth and germination of Cercospora beticola.

Benomyl, however, appears to have no inhibitory effects on germination, even at high concentrations. Decallone and Meyer (1972) reported that benomyl did not prevent germination in Fusarium oxysporum but merely reduced the rate of germ tube formation. Likewise, early observation of

N. crassa treated with MBC indicated that conidia had germinated but hyphae had become large and misshapen (Clemons and Sisler, 1971). These results suggest that both benomyl and MBC function to impede elongation rather germination.

Benomyl and MBC produce different effects when added to cultures of Saccharomyces pastorianus (Hammerschlag and Sisler, 1972). The addition of benomyl caused a decrease in growth but no morphological aberrations were observed and cells were viable. On the other hand, the same amounts of MBC permitted some growth but daughter cells failed to separate and resultant cell clusters appeared containing enlarged cells, some of which were terminally elongated. Viability of these cells was very low.

Cytological Effects

The prevalence of swollen and distorted germ tubes of sensitive fungi treated with thiabendazole, benomyl and MBC was acknowledged in the discussion above. However, seldom has there been more than a passing reference to the cytological malformations which materialized. Richmond and Pring (1971) have provided excellent photomicrographs and electron micrographs which clearly illustrate modification in the ultrastructure of Botrytis fabae grown in the presence of sublethal amounts of benomyl. They verified the production of misshapen germ tubes and also detected more frequent branching of young hyphae from treated conidia. An increase in multiple germ tube emergence was likewise established,

and the changes induced by the toxicant were reversed when germinating conidia were transferred to benomyl-free medium. Thin sections and freeze-fractured replicas revealed alterations in endoplasmic reticulum such that concentric sheets in normal germ tubes appeared as a branched network in treated cells. Some strands of endoplasmic reticulum terminating in vesicles were near the plasma membrane, and the authors suggested these vesicles might play a role in the multiple branching produced by benomyl treatment. The observed increase in lomasomes was probably a reflection of modification in the endoplasmic reticulum. Thin sections of nuclei did not disclose any differences between treated and untreated conidia, but surfaces of freeze-etched nuclei in treated cells were deeply lobed and convoluted suggesting some action of benomyl on the nucleus. Other organelles were not affected and cell wall thickness was not changed

In the electron micrographs prepared by Say (1970) on S. cerevisiae grown in the presence of 100 µg/ml benomyl, gross transformations in mitochondrial structure were detected. Treated cells possessed only a few small organelles resembling typical mitochondria, and none were as well differentiated as those in normal cells. Say's results imply that the mitochondria are the targets of benomyl inhibition in yeast.

Respiration

Slonimski (1954, 1956) observed that benzimidazole suppressed the induced biosynthesis of a functional respiratory

system in yeast cells. More specifically, he found that when cells previously grown anaerobically in galactose and benzimidazole were grown aerobically with carbohydrates, adaptability (measured by O_2 uptake) was drastically reduced. Sels confirmed these results and postulated that benzimidazole interfered with assembly of respiratory apoproteins for the formation of functional holoenzymes. Hence, in the presence of the toxicant, catalytically inactive enzyme precursors accumulated.

The effect of thiabendazole on respiration in P. atrovenetum was elucidated by Allen and Gottlieb (1970). Their experiments with the fungus and with beef heart as well, localized the site of inhibition between succinate and cytochrome c. Further work demonstrated that succinic:cytochrome c reductase was inhibited by low concentrations of thiabendazole, but cytochrome c oxidase was unaffected. Interference with reduction of coenzyme Q_6 by succinate indicated the action of the fungicide was between succinate and coenzyme Q. In contrast, thiabendazole had no effect on oxygen utilization by the Basidiomycete, U. maydis (Hammerschlag and Sisler, 1973).

In studies on the differential action of benomyl and MBC on S. pastorianus, Hammerschlag and Sisler (1972) noticed that benomyl inhibited glucose and acetate oxidation; at low levels of the toxicant recovery occurred within 5 hours after treatment. MBC, on the other hand, did not alter respiration with either glucose or acetate as substrates. These results

were substantiated when MBC was found to have no effects on glucose oxidation in S. cerevisiae and U. maydis. However, oxygen uptake was inhibited by benomyl in both organisms (Hammerschlag and Sisler, 1973). The levels of inhibition were dependent upon the ratio of toxicant concentration to the number of cells present. This is not surprising since it is reasonable that the quantity of toxicant taken up by the cells is determined by the amount of toxicant per unit weight of cells (Miller, 1959).

Hammerschlag and Sisler (1973) have proposed that one of the products of benomyl degradation, in addition to MBC, is butyl isocyanate. An aqueous solution of benomyl was made and after standing 8 hours a compound extracted in hexane was identified by gas-liquid chromatography and infrared spectroscopy as butyl isocyanate. These authors found that butyl isocyanate inhibited respiration in both S. cerevisiae and U. maydis in a manner similar to benomyl; thus the anti-respiratory activity observed with benomyl was attributed to the butyl isocyanate moiety which may be formed in cells treated with the parent compound. The formation of butyl isocyanate may explain the differential toxicity of MBC and benomyl on respiration seen earlier in S. pastorianus (Hammerschlag and Sisler, 1972), and the breakdown of butyl isocyanate to other non-toxic components (i.e., n-butylamine) may account for the recovery which occurred after five hours.

The detrimental consequences of benomyl on respiration in S. cerevisiae were also substantiated by the work of Say (1970). With glucose as a substrate, she was able to demonstrate 50% reduction in oxygen uptake with benomyl (6 and 12 μg/ml), whereas growth of this organism was inhibited only by concentrations greater than 50 μg/ml. Benomyl-grown cells were found to have an impaired respiratory rate which was still sensitive to the effects of benomyl.

Benomyl likewise suppressed respiration in F. oxysporum (Decallone and Meyer, 1972). Among several enzymes which were tested, only L-malate:NAD oxidoreductase was sensitive to the fungicide. The authors did not feel the primary mechanism of action of benomyl was the inhibition of respiration since levels of the toxicant necessary to decrease respiratory activity were much higher than those which affected growth.

In organisms other than those discussed above, i.e., Phymatotrichum omnivorum (Lyda and Burnett, 1970) and Aspergillus niger (Kaars Sijpesteijn, 1970), amounts of benomyl much greater than those required to halt growth produced no change in respiration. Clemons and Sisler (1971) found no effect by MBC on glucose oxidation in N. crassa.

To date no reports have appeared which deal with the possible role of benzimidazole fungicides in uncoupling oxidative phosphorylation. Jones and Watson (1967) made a survey of the activity of 25 substituted benzimidazoles in uncoupling. Their results showed that: 1) with halogen,

mixed halogen and alkyl analogues, uncoupling was proportional to the acidity of the imidazole group, 2) loss of uncoupling properties coincided with addition of $-NH_2$ or -COOH substituents at the 5 position or with alkylation at position 1 of the imidazole ring, 3) there was no evidence of complex formation between benzimidazoles and non-heme iron or heme enzymes, and 4) of all benzimidazole derivatives tested, 4,5-dichloro-2-trifluoromethyl benzimidazole was most active. In view of these results there is no definitive reason why any of the benzimidazole fungicides should be inhibitors of oxidative phosphorylation.

Metabolism and Biosynthesis

When Woolley (1944) recognized the reversal of benzimidazole-inhibited growth in microorganisms by adenine and guanine, he theorized that benzimidazole might function as a purine analogue. Other purines (xanthine and hypoxanthine), pyrimidines (uracil) and nucleotides (adenylic acid) were ineffective in reversing benzimidazole inhibition of yeast and *E. coli*. In *S. lactis*, however, uracil was the most antagonistic. Therefore, it seems conceivable that benzimidazole does interact in some way with the pathways of nucleotide synthesis, although it is not clear just how. It is interesting to note that compounds which would be more analogous to adenine or guanine, such as 4-aminobenzimidazole and 4-nitrobenzimidazole, had approximately the same potency as unsubstituted benzimidazole, and 5-aminobenzimidazole was

one-half as active (Woolley, 1944).

Bartels-Schooley and MacNeill (1971) have published information pertaining to the reversal of benomyl, thiabendazole and furidazole inhibition in F. oxysporum. Fungitoxicity of all three compounds was relieved by adenine, guanine, hypoxanthine, xanthine, aspartic acid and biotin at pH 8.5. At pH 6, however, only adenine, aspartic acid and biotin were effective. The inferences drawn from this data were: 1) all three compounds act in a similar manner by competing with free purines and preformed nucleotides, 2) reversal by aspartic acid is due to enhancement of inosinic acid synthesis (the effect of biotin is indirect and would increase the amount of aspartic acid made), and 3) the differential antagonism exhibited by metabolites at pH 6 undoubtedly reflects the relative affinities of individual fungicides for the enzymes involved.

The possibility that benzimidazole may well be a purine analogue is most clearly illustrated by the effects of benzimidazole on plant tissues. The fact that benzimidazole retards the senescence of detached leaves has been noticed by several investigators (Person et al., 1957; Wang et al., 1961; Kim, 1970; Yoshida, 1970). The phenomenon is not well understood and several theories have been advanced to explain it, one of which is that benzimidazole causes an increase in the ratio of NADP to NAD (Mishra and Waygood, 1968; Godavari and Waygood, 1970). NAD is known to accelerate the senescence of detached wheat leaves (Yoshida, 1970), an effect

reversible by benzimidazole but not by NADP.

In their studies on the mechanism of action of benzimidazole in detached wheat leaves, Kapoor and Waygood (1965a) demonstrated the presence of an enzyme, benzimidazole nucleotide:pyrophosphate phosphoribosyl transferase (benzimidazole nucleotide pyrophosphorylase) which catalyzed the reaction:

The enzyme could be assayed indirectly due to the competitive inhibition between benzimidazole and orotic acid for the orotidine-5'-phosphate pyrophosphorylase, which was isolated in the same fraction. Incubation of C^{14}-benzimidazole with wheat embryo homogenates yielded a product identified by electrophoresis, chromatography and absorption spectrum as benzimidazole nucleotide. The formation of this benzimidazole nucleotide may have many ramifications in the metabolism of cells. Friedman and Harris (1962) showed that nucleosides of benzimidazole and 5,6-dimethylbenzimidazole are precursors to synthesis of vitamin B_{12} in Propionibacterium shermanii, and Fries (Kapoor and Waygood, 1965a) has speculated that benzimidazole is incorporated into a vitamin B_{12}-like factor in wheat leaves. It is also possible benzimidazole nucleotide may compete with adenine nucleotide in the formation of nucleic acids.

Kapoor and Waygood (1965b) have suggested that benzimidazole has a role in metabolism of nicotinamide nucleotides. A particulate fraction of wheat leaves which contained a glycohydrolase (NAD nucleosidase) catalyzed the cleavage of the pyridinum N-ribosyl linkage and also the substitution of benzimidazole for the nicotinamide moiety of NAD. Analysis of products formed from a reaction mixture containing benzimidazole revealed the presence of benzimidazole nucleoside. Based on their observations, the authors proposed the following mechanism:

benzimidazole + NAD —glycohydrolase→ benzimidazole adenine dinucleotide + nicotinamide

benzimidazole adenine dinucleotide —nucleotidase→ benzimidazole nucleoside + NAD

It has not yet been demonstrated whether benzimidazole adenine nucleotide is phosphorylated to become an analogue of NADP.

Allen and Gottlieb (1970) investigated some of the metabolic effects of thiabendazole (other than respiratory effects) in P. atrovenetum. At concentrations of thiabendazole which inhibited growth, little change was observed in synthesis of lipids, proteins, nucleic acids, nucleotides and carbohydrates, as measured by incorporation of C^{14}-amino acids into various fractions extracted from cells (i.e.,

trichloroacetic acid, chloroform:methanol, etc.). Allen and Gottlieb (1970) also noticed that *in vitro* protein synthesis in *Rhizoctonia solani* was not altered by the addition of thiabendazole.

The effects of benomyl (and MBC) on biosynthesis have been more extensive and more contradictory than thiabendazole. Table I summarizes what is now known concerning the inhibition of DNA, RNA and protein synthesis induced by benomyl as well as MBC.

Incorporation of C^{14}-uracil into total RNA of *F. oxysporum* was inhibited after incubation in benomyl but incorporation of C^{14}-lysine into protein was not affected (Decallone and Meyer, 1972). It appears, therefore, that in this organism benomyl partially prevents *de novo* RNA synthesis. Such an effect has been reported for benzimidazole (Kapoor and Waygood, 1965a) which was shown to compete with orotic acid for orotidine-5'-phosphate pyrophosphorylase as previously mentioned. However, Decallone and Meyer (1972) failed to see any competition between benomyl and orotic acid.

Synthesis of both DNA and RNA as measured by C^{14}-uridine incorporation was almost immediately terminated by benomyl in *S. pastorianus*. Treatment with MBC caused a reduction in DNA synthesis but only after a lag period of 1 hour. RNA synthesis was decreased only 40%. MBC did not appreciably affect incorporation of C^{14}-phenylalanine until after 3 hours (Hammerschlag and Sisler, 1972).

Table 1. The effects of benomyl and MBC on nucleic acid and protein synthesis in fungi and yeasts. Numbers in parentheses represent concentration of toxicant (in μg/ml) at which inhibition was observed.

Organism	Time course of experiment (hours)	% Inhibition						Reference
		DNA		RNA		Protein		
		Benomyl	MBC	Benomyl	MBC	Benomyl	MBC	
S. pastorianus	4	95(10)	95(10)*	95(10)	50(10)	95(10)	70(10)	Hammerschlag and Sisler, 1972
U. maydis	3		96(8)		87(8)*		65(8)*	Clemons and Sisler, 1971
N. crassa	4		45(8)*		0(8)		0(8)	Clemons and Sisler, 1971
	6		84(8)*		18(8)		0(8)	
S. cerevisiae	4		0(5)					Hammerschlag and Sisler, 1971
A. nidulans	8		100(0.8)*		100(0.8)*			Davidse, 1973
F. oxysporum	7			75(20)		0(20)		Decallone and Meyer, 1972

*Time lag before onset of inhibition

Using a similar method of measuring DNA, RNA and protein synthesis, Clemons and Sisler (1971) studied the effect of MBC on U. maydis and N. crassa. In U. maydis strong inhibition of DNA and RNA synthesis was observed although there was a lag in the onset of inhibition of RNA synthesis. The same level of MBC also reduced incorporation of C^{14}-phenylalanine into protein after a 1-hour lag. In N. crassa DNA synthesis was not affected until 3 hours following addition of benomyl; after that time, incorporation progressively decreased to 16% of control. The rate of RNA synthesis was only slightly inhibited and protein synthesis was unaffected.

Experiments with synchronous cultures of S. cerevisiae (Hammerschlag and Sisler, 1972) revealed that cells grown in the presence of MBC were able to double the initial amount of DNA, but further replication was inhibited. Microscopic examination disclosed that treated cells had remained fixed as doublets (joined mother and daughter cells), whereas control cells had separated and continued to produce new DNA. This disclosure suggests MBC does not affect DNA directly and that inhibition of DNA synthesis is secondary. Similar results were obtained using MBC in synchronous cultures of U. maydis. Doublets appeared within the first 2.5 hours of incubation and then began to separate in control cultures followed by replication of DNA in the separated cells. Treated cells, however, failed to divide and the amount of DNA present remained the same.

Davidse (1973) has elucidated some important information concerning the effect of MBC on nucleic acid synthesis in Aspergillus nidulans mycelia of different ages. He found that synthesis of DNA and RNA in 10 hour-old cultures was inhibited only 43% and 13% respectively. Inhibition of RNA showed a 1-hour lag behind observed reduction in DNA content. In 16-hour cultures there was nearly 100% decrease of both DNA and RNA. The effect of MBC on RNA synthesis was explained by the author. In 10 hour-old hyphae, the RNA content per mg dry weight is decreasing, therefore RNA synthesis is less inhibited than DNA synthesis. In 16-hour hyphae, RNA and DNA syntheses were suppressed to the same extent, indicating a closer correlation between DNA and RNA content.

It might be mentioned here that several other benzimidazole derivatives have been implicated as inhibitors of nucleic acid synthesis. Egyhezi et al. (1970) used 5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole to prevent RNA synthesis in salivary glands of Chironomus tentans. The toxicant radically reduced labeling with cytidine and uridine in heterogenous chromosomal RNA while other RNA species (low molecular weight RNA, 4-5S RNA and nucleolar RNA) were only partially affected. It was suggested that this compound may inactivate one of the RNA polymerases or act as an antagonist to purine nucleotides.

Bucknall (1967) first studied the effects of several substituted benzimidazoles on RNA metabolism in calf kidney and mouse L-cells, and then measured their ability to support

RNA and DNA viruses. Compounds tested were 4,5,6-trichloro-1-β-D-ribofuranosyl benzimidazole, 2-mercapto-1-(β-4-pyridethyl) benzimidazole and 2-(β-hydroxybenzyl) benzimidazole. Both 4,5,6-trichloro-1-β-D-ribofuranosyl benzimidazole and 2-mercapto-1-(β-4-pyridethyl) benzimidazole suppressed DNA and RNA synthesis in both types of cells and treated cells were not able to support viral replication. While 2-(β-hydroxybenzyl) benzimidazole had little or no effect on nucleic acid production by host cells, it did prevent viral growth. Presumably, this compound is specific for viral induced RNA synthesis, perhaps by interference with the formation of a viral coded polymerase.

Bucknall and Carter (1967) showed that inhibition of RNA and DNA in mouse L-cells by 2-mercapto-1-(β-4-pyridethyl) benzimidazole was reversed by addition of adenosine and guanosine but not orotic acid, cytidine or dimethyl-ribofuranosyl benzimidazole, a component of vitamin B_{12}. By separation of RNA species, they found both tRNA and rRNA syntheses had been inhibited. Subsequent experiments proved that 2-mercapto-1-(β-4-pyridethyl) benzimidazole had no activity against RNA polymerase or nucleotide kinases, but, in fact, inhibited uptake of nucleosides in both normal and tumor cells (Nakata and Bader, 1969).

Cell Division

The information concerning the impact of benzimidazoles on mitosis is conflicting. Gracza and Pozsar (1970), in

examining root development in bean plants, found a concentration-dependent inhibition by benzimidazole on main and lateral root formation. The compound inhibited cell division and elongation 100% at a concentration of 100 μg/ml.

Clemons and Sisler (1970) were the first to recognize the possibility that MBC might act on nuclear and/or cell division in fungi. Since the DNA content of N. crassa conidia increased prior to inhibition of synthesis, interference with spindle formation or mitosis was considered. The results obtained with U. maydis (i.e., the immediate inhibition of DNA) did not lend support to such a mechanism. However, the appearance of clusters of enlarged cells with low viability in cultures of MBC treated S. pastorianus led Hammerschlag and Sisler (1972) to believe cell division had indeed been affected. Cells grown in benomyl for a short time did not show these effects, perhaps because no division was initiated. In long term experiments benomyl treated cells recovered; morphology and viability were normal.

In 1973, Hammerschlag and Sisler explored the effects of MBC and benomyl on U. maydis and S. cerevisiae. As was discussed in the previous section, MBC-treated yeast cells went through one round of DNA replication just before mitosis, but became fixed as doublets. Microscopy of stained cells disclosed that growth had terminated with a doublet containing a single nucleus, hence mitosis was incomplete. Experiments with U. maydis demonstrated that essentially the same response had occurred, as MBC added at any time prior

to the onset of cell division prevented completion of that division. The search for possible effects on the mitotic spindle was unsuccessful. If benomyl did act at this site, however, it might explain the reversal of benomyl inhibition in S. cerevisiae by thiols (Mailman et al., 1971).

Davidse (1973) has also concluded that MBC may interfere with mitotic division. Upon addition of MBC to 10-hour cultures of A. nidulans, the number of nuclei per germ tube remained the same, as opposed to a rapid increase in number in control cells. DNA synthesis was inhibited after one hour but the calculated DNA content per nucleus rose sharply to nearly twice the initial content after 4 hours. Stained hyphae revealed that chromatin in treated cells was contracted, clumped and unevenly distributed while in control cells the chromatin had a ring-shaped appearance. These results may indicate that MBC does arrest mitotic activity and that inhibition of DNA synthesis is an indirect consequence.

Cytogenic and Mutagenic Effects

Boyle (1973) attempted to determine cytogenic effects of benomyl on chromosomes of Alluim cepa and Secale cerale. The only aberrations located were lagging chromosome fragments in mitotic cells; these fragments were present at very low frequency and at concentrations of benomyl higher than recommended field application. No effect on meiosis was established.

Benomyl treatment of diploid strains of A. nidulans did produce a marked increase in the emergence of haploid segregants (Hastie, 1970). Segregational events in the presence of benomyl apparently took place early in the growth of the colony due to the large areas occupied by the segregants. Hastie could not find any evidence of mutational events resulting from exposure to the toxicant, but either chromosome breakage or spindle interference might cause the instability of Aspergillus diploids.

Seiler (1972, 1973a) analyzed the mutagenicity of benzimidazole and several derivatives by testing for forward mutations in Salmonella typhimurium. The compound, 2-aminobenzimidazole, was found to be weakly mutagenic; benzimidazole, MBC and furidazole were "relatively" mutagenic (i.e., 5-10 mutations per 10^8 cells). Using various histidine auxotrophs containing either a base substitution or a deletion or insertion, Seiler measured reversion frequency in the presence of benzimidazoles. Revertants occurred only in those strains with base substitutions; hence mutation caused by these agents must have been base substitutions. In an effort to prove this theory, Seiler (1973b) isolated and hydrolyzed DNA and RNA from E. coli grown with benzimidazole and P^{32}. Electrophoresis of the hydrolysate revealed the existence of an additional band when compared to a hydrolysate from control cells. The mobility of the new band corresponded with that of a synthesized benzimidazole

nucleotide. Seiler concluded that benzimidazole acts as a mutagen by replacing a base and may further result in a transition or transversion.

Dassenoy and Meyer (1973) were able to isolate 4 kinds of auxotrophic mutants from conidia of F. oxysporum treated with benomyl at 5 µg/ml. No spontaneous nutritional mutants were obtained. Apparently the mutagenic action of benomyl was manifested only in growing cells, since no auxotrophs were selected from benomyl treated conidia in saline.

MATERIALS AND METHODS

Strains

Neurospora crassa wild type strains studied throughout this investigation were 74-OR8-1a (FGSC# 988) and 74-OR23-1A (FGSC# 987), both of St. Lawrence background. Strain SF26 (Gratzner and Sheehan, 1969) was used in some experiments concerning the effects of benomyl on growth, protein synthesis, invertase secretion and cell wall synthesis. *Slime* (FGSC# 1118) was also employed in certain studies. The auxotrophic strains which were used for specific purposes carried the following requirements: histidine (*hist-1*, FGSC# 681), adenine (*ad-1*, FGSC# 672), pyrimidine (*pyr-3*, FGSC# 835) and leucine (*leu-3*, FGSC# 1124). Strains utilized in genetic mapping of benomyl resistant mutants were *fluffy*^{P} (FGSC#'s 1690, 1838), *alcoy* (FGSC#'s 997, 998), *tryp-1;ylo-1* (FGSC#'s 1207, 1208) and *tryp-2;ylo-1;chol-2* (FGSC#'s 2091, 2092). All stock cultures were provided by the Fungal Genetics Stock Center, Humboldt State College Foundation, Arcata, California. *Fluffy*^{P} was furnished by Dr. D. R. Parker, Stanford University. All cultures except *fluffy*^{P} were stored on silica gel (Perkins, 1962) at 4 C.

Chemicals

Glucostat reagent and chitinase (*Streptymyces griseus*) were obtained from Worthington Biochemical Corp.,

diphenylamine from Fisher Scientific Co., and orcinol from Matheson, Coleman and Bell. Orcinol was recrystallized before use according to the procedure described by Schneider (1957). DNA (salmon sperm) and RNA (Torula) were provided by Cal Biochemicals. Aquasol universal liquid scintillation cocktail and C^{14}-leucine (uniformly labeled) were purchased from New England Nuclear. Reeve Angel was the source of DEAE-cellulose (Whatman DE-23). Adenine-8-C^{14} and uridine-5-H^3 were both provided by Amersham/Searle Corp. Technical grade benomyl [methyl-1-(butylcarbamoyl)-2-benzimidazole] was supplied by E. I. duPont de Nemours and Co. All other chemicals were reagent grade and were obtained from commercial sources.

Media

Cultures were maintained and experiments carried out using Vogel's citrate minimal salts solution (1956) containing 1.5% sucrose; in addition, solid media contained 1.5% agar. Where auxotrophic mutants were employed, growth supplements were added in the following concentrations: adenine sulfate, 100 µg/ml; uridine, 100 µg/ml; choline chloride, 30 µg/ml; indole, 20 µg/ml; tryptophan, 150 µg/ml; histidine HCl, 100 µg/ml. Uridine was used as a supplement for pyr-3; tryp-1 mutants were grown on minimal medium plus indole, and medium for tryp-2 mutants was enriched with tryptophan. All crosses were carried out on the medium recommended by Westergaard and Mitchell (1947) with 2% sucrose and required growth

factors.

When it was desirable to induce colonial growth of Neurospora, a sorbose medium was employed, composed of minimal medium with 1% sorbose and 0.1% sucrose (or 0.025% glucose and 0.025% fructose). The complete medium used in some experiments was sorbose medium supplemented with 0.5% yeast extract and 1% casamino acids. The media described by Bigger et al. (1972) were utilized for slime cultures.

General Procedures

Source Cultures

Cultures employed were maintained by periodic transfer of conidia or vegetative growth to slants of the appropriate medium. They were incubated at 34 C, then production of conidia was induced by allowing cultures to remain at room temperature in the light for at least 2-3 days.

Inocula

Conidial suspensions were used as the inoculum for all experiments carried out in liquid media. The suspensions were routinely prepared by adding sterile water to slants or flasks containing 5-day old cultures of Neurospora. After shaking, the liquid was filtered through 3 layers of cheesecloth to remove mycelial fragments. Equal aliquots of the filtered suspension were added to control and benomyl media. When a standardized inoculum was desired, the spore concentration was calculated by counting a dilution of the conidial

suspension in a hemocytometer.

Liquid slime cultures were inoculated by the following procedure: a small amount of sterile broth medium was added to each slant of slime and cells were carefully scraped from the surface of the agar. Broth suspensions from several tubes were pooled and gently homogenized by pipetting. Equivalent volumes were then aseptically transferred to each flask.

Addition of Benomyl

Benomyl was dissolved in 100% ethanol or 100% methanol immediately before use and added to the autoclaved medium using standard aseptic procedures. The volume of benomyl solution added never exceeded 0.1% (v/v) of the medium.

Growth of Neurospora in Large Quantities

Bulk quantities of Neurospora used for amino acid and cell wall analysis and for determination of nucleotide pools were obtained in the following manner. Two 9-liter volumes of Vogel's minimal salt solution were autoclaved in 12-liter carboys for 1 hour. Upon cooling, 1 liter of a sterile 10% sucrose solution was added. Both carboys were inoculated with 100 ml conidial suspension of strain SF26 and incubated with aeration at 34 C. After 6 hours of growth, benomyl was added to one of the carboys to give a final concentration of 1.5 ug/ml and an equivalent amount of ethanol was added to the control. Growth was allowed to continue for 48 more hours.

Mycelia from the control culture were filtered through 2 layers of cheesecloth, washed with ice cold water and placed immediately on ice. Benomyl treated Neurospora was filtered through Whatman #1 filter paper in a large Buchner funnel, rinsed and placed on ice. Both samples were then frozen, lyophilized and weighed. Untreated mycelia were ground to a fine powder in a Wiley mill, whereas treated cells were ground in a mortar and pestle. Mycelial powders were stored at 4 C until use.

Growth Curves

Solid medium. Growth rates in the presence and absence of benomyl were measured two ways. A small amount of Neurospora conidia was transferred to solid medium containing varying concentrations of benomyl, and mycelial growth was evaluated at 34 C by the method of Ryan et al. (1943).

Liquid medium. Growth of SF26, pyr-3 and ad-1 was also followed in liquid medium. Nephelometer flasks containing minimal or supplemented media and various amounts of benomyl were inoculated and allowed to incubate at 30 C on a rotary shaker. The quantity of cell material present was estimated periodically on a Klett-Summerson colorimeter using filter #54.

The effect of benomyl on slime was assayed in a similar manner except that readings were taken using filter #66.

Assay Methods

Protein concentration was determined by the method of

Lowry et al. (1951) or by absorbance at 280 nm. Total hexose was measured according to the procedure of Dubois et al. (Ashwell, 1966) with glucose as a standard. Hexosamine was estimated by a modified Elson and Morgan method (Davidson, 1966). Glucostat reagent was employed in the assay for glucose. Determinations of extra-labile, labile and stable phosphorus were made using the technique of Fiske and SubbaRow (Leloir and Cardini, 1957). Orcinol and diphenylamine reagents were employed in the evaluation of RNA and DNA, respectively (Schneider, 1957).

Amino Acid Analysis

Two mg of lyophilized mycelial powder or crude cell wall preparation were placed in tubes with 2 ml of 6 N HCl. The tubes were sealed under vacuum and samples were hydrolyzed at 100 C for 24 hours. After the tubes were opened, the samples were dried in a vacuum desiccator over NaOH and H_2SO_4. Dried samples were dissolved in 1 ml of 0.2 M citrate buffer, pH 2.2, and then 0.1 ml of the solution was used for amino acid analysis. The techniques of Spackman et al. (1958) were followed for the analyses on a Beckman Spinco Model 120C amino acid analyzer.

Hydrolysis for Carbohydrate Determination

Two ml of 3 N HCl were added to 2 mg of lyophilized mycelial powder or cell wall preparation. Tubes were sealed under vacuum and the contents were hydrolyzed for 3 or 6 hours at 100 C. The tubes were opened and dried under vacuum

with NaOH and H_2SO_4. Samples were then redissolved in 2 ml of distilled water.

Cytological Methods

Light Microscopy

The effects of benomyl on morphology and development were illustrated in the following way: benomyl (or an equivalent amount of methanol) was added to 50 ml cultures of 74A at the time of inoculation, and then cultures were incubated at 34 C on a rotary shaker. Aliquots were aseptically removed at 1, 2, 4, 6, 8 and 12 hours after inoculation. Wet mounts were made from each culture and photomicrographs were taken using a Bausch and Lomb microscope with an oil immersion lens.

Electron Microscopy

Neurospora SF26 was grown in minimal medium containing 2% mannitol (to maintain isotonic conditions) for 6 hours at 30 C. Benomyl was added to give final concentrations of 0.2, 0.5 and 1.5 µg/ml. Cultures were further incubated for 24 hours. Mycelia were harvested by centrifugation and fixed in 3% glutaraldehyde plus 5% sucrose in Sorenson's (1909) phosphate buffer for 2 to 4 hours at 4 C. Cells were then washed overnight at 4 C in Sorenson's buffer containing 5% sucrose. Post fixation was carried out for 1½ to 2 hours in OsO_4 with 5% sucrose in Sorenson's buffer. Following dehydration with graded concentrations of ethanol, the cells were infiltrated

with ethanol-Spurr and Spurr alone. Embedding was done in fresh Spurr and capsules were polymerized at 70 C for 8 hours. Sections of silver or silver-gray interference colors were cut on an LKB microtome using a Dupont diamond knife. They were post-stained with saturated uranyl acetate in 95% ethanol and then with Reynold's lead citrate. Sections were examined on an RCA EMU-3-G electron microscope.

Slime cultures were allowed to incubate at 30 C on a rotary shaker for 72 hours, after which benomyl was added to give 0.2 and 0.5 ug/ml. Incubation was continued for an additional 12 hours, cells were collected by centrifugation, and fixed, embedded and stained according to procedures described by Bigger et al. (1972).

Experimental Outline

Membrane Permeability

Cultures of wild type Neurospora were incubated for 6 hours at 30 C and benomyl was added to one flask to give 1 µg/ml final concentration. After 8 and 24 hours, aliquots were removed from the control and treated cultures and centrifuged to sediment mycelia. The absorbance of the supernatant fluid (media) was measured at 260 nm and 280 nm and then assays for labile phosphorus were performed on these aliquots. Cultures of slime were used in a similar experiment except that benomyl was added at 8 hours, and aliquots were removed immediately after benomyl was added and then after 8 and 24 more hours. Only absorbance was measured on

these samples.

Cell Wall Analysis

To determine the effect of benomyl on cell wall synthesis, 1.5 grams of lyophilized mycelial powder from control and treated cultures was treated for isolation and fractionation of cell wall material (Mahadevan and Tatum, 1965). Fractions were lyophilized, weighed and analyzed for content of protein, total hexose, glucose, hexosamine and acetyl hexosamine. For comparative purposes, whole mycelial powder, crude cell wall material and several samples of fractionated cell wall were subjected to hydrolysis prior to carbohydrate and amino acid analysis. Fraction IV (chitin) was also treated with chitinase. Five mg of fraction IV were placed in a flask containing 1 unit (0.025 mg) of chitinase in 25 ml of 0.05 M phosphate buffer, pH 5.0. This mixture was incubated on a rotary shaker at 37 C for 48 hours. The liquid was then filtered to remove undigested debris and assayed for concentration of N-acetyl glucosamine.

Invertase Production

Four flasks containing minimal media were inoculated with 74A conidia. One flask served as a control, a second had benomyl (1.5 µg/ml) added at the time of inoculation and a third had benomyl (1.5 µg/ml) added 6 hours after inoculation. Cultures were incubated 36 hours at 34 C on a rotary shaker. Mycelia were filtered, washed, lyophilized and weighed.

Invertase was extracted from 0.05 g mycelial powder by stirring in 0.075 M acetate buffer (pH 4.2) for 1½ hours at 4 C. This suspension was centrifuged to sediment mycelia; the supernatant was removed and assayed for invertase activity by the method of Metzenberg (1962) as modified by Meachum et al. (1971).

Reversal of Inhibition

Attempts to reverse the inhibitory effects of benomyl were carried out in two ways. Flasks were prepared such that they contained: a) liquid minimal media, b) media plus supplement (100 µg/ml), c) media plus benomyl (0.5 µg/ml), d) media plus benomyl and supplement (100 µg/ml). Each flask was inoculated with conidia from 74A and allowed to incubate on a rotary shaker at 30 C. After 48 hours cultures were filtered, washed with distilled water, lyophilized and weighed.

In another experiment, plates were prepared with solid media and 1 µg/ml benomyl. Plates were smeared with equal amounts of a conidial suspension of 74A and then sterile solutions of test compounds were added dropwise to give varying concentrations. Plates were incubated at 34 C for 2 days and observed for growth.

For a third type of experiment, plates were prepared as outlined above. Aliquots of test solutions were placed on the surface of the agar and allowed to penetrate. When the surface was dry, one loopful of conidial suspension was added

to the center of the agar plate. Plates were incubated at 34 C for 12 hours and the distance was measured from the point of inoculation to the perimeter of mycelial growth.

Incorporation Experiments

Wild type strain 74A was used to measure incorporation of C^{14}-leucine, C^{14}-adenine and H^{3}-uridine into protein and nucleic acids, respectively. Conidia were added to 25 or 50 ml of media to give 1-2 x 10^{6} conidia/ml of medium. The cultures were incubated on a rotary shaker at 34 C for 6 hours. At the end of this period benomyl in methanol was pipetted into one-half the number of flasks to give a final concentration of 1 µg/ml. Cultures were incubated for 5 minutes and then labeled precursor was added to give approximately 3000 cpm/ml of media. At 2 minutes, 5 minutes, 30 minutes, 1 hour, and 2 hours after addition of label, one flask each of control and treated cultures were removed and their metabolism terminated by addition of cold trichloroacetic acid (TCA) to give 5% final concentration. Mycelia were filtered, washed thoroughly with 2 to 3 volumes of water, lyophilized and weighed. Dried samples were homogenized for 1 minute in cold TCA in a Sorvall Omnimixer set at maximum speed. The homogenates were then centrifuged at 27,000 x g to remove precipitated protein and nucleic acid and then supernatant removed. Pellets were washed with 5% TCA; supernatant and wash were pooled and aliquots removed for radioactive counting in Aquasol using a Beckman TriCarb

Liquid Scintillation Counter, Model LS200. The pellets were resuspended in 95% ethanol and incubated at 40-50 C for 30 minutes. After centrifugation and removal of the supernatant, the pellets were resuspended in ethanol:ether (1:1) for 15 minutes at 40-50 C. This was centrifuged and supernatant discarded. When protein was to be determined, pellets were resuspended in 5% TCA for 1 hour at 90 C to remove nucleic acid, centrifuged and then washed with acidified alcohol followed by ether. Protein was extracted from the pellet with 1 ml of 1 N NaOH at 40 C for 15 minutes.

To measure nucleic acids, the ethanol:ether pellet described above was incubated in 0.5 N NaOH (2 ml) for 18 hours at 37 C to hydrolyze RNA. Subsequent to addition of 0.5 ml of 6 N HCl and 1 ml of 5% TCA to precipitate DNA, the samples were centrifuged and the supernatant (hydrolyzed RNA) removed. DNA was extracted thrice from the resulting pellet with 1 ml of hot 5% TCA. Both hydrolyzed RNA and the DNA fraction were counted and assayed colorimetrically. All results are reported as cpm of labeled material incorporated per mg of protein or nucleic acid.

In an effort to obtain greater levels of incorporation into nucleic acid, mutant strains ad-1 (adenine requiring) and pyr-3 (supplemented with uridine) were used in experiments exactly like those previously described. Media for these experiments were enriched with 20 µg/ml cold adenine sulfate (ad-1) or 20 µg/ml cold uridine (pyr-3).

Double-Labeling Experiments

Column preparation. DEAE-cellulose was precycled by first suspending in 15 volumes of 0.5 N HCl for 30-40 minutes. The suspension was filtered and washed until the effluent reached pH 4. The exchanger was then suspended in 15 volumes of 0.5 N NaOH for 30-40 minutes and then filtered. More 0.5 N NaOH was added for the same length of time, after which the exchanger was filtered and washed until the effluent pH was near neutral. Equilibration was carried out by adding 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer and titrating to pH 7.2. The exchanger was then filtered and washed 4-6 times with 0.01 M Tris buffer, pH 7.2 until the pH remained constant. Fines were removed by decanting, then the exchanger was degassed and the column packed in Tris buffer. The column was washed with 2-3 volumes of buffer and the pH of the effluent was monitored before applying the sample.

Experimental procedure. Flasks containing 250 ml of media were inoculated with conidia from 74A to give 1.5 x 10^6 conidia/ml and cultures were incubated on a rotary shaker for 6 hours at 34 C. Benomyl was added to one-half the number of flasks and incubated for 5 minutes. H^3-uridine and C^{14}-adenine were pipetted into each culture to give approximately 3000 cpm/ml of medium. One control and one treated culture were removed 30 minutes, 2 hours, 4 hours and 8 hours after addition of labeled material. Reactions were terminated by adding cold trichloroacetic acid; mycelia were

filtered, washed extensively with water to remove adsorbed label, lyophilized and weighed. The volumes of the filtered media were recorded and aliquots taken for counting to determine the amount of label remaining. Then, equal amounts of lyophilized mycelia were homogenized for 2 minutes in 0.01 Tris buffer (pH 7.2) using an Omnimixer. The total volume of homogenates was brought to 15 ml and three 0.1-ml aliquots were removed for counting. Homogenates were deproteinized by extraction with 15 ml of 0.01 M Tris saturated with phenol for 2 hours at 25 C. The phenol was separated by centrifugation at 5,900 x g. The upper aqueous layer was removed and adjusted to 25 ml total volume whereas the phenol portion was adjusted to 11 ml total. Aliquots (0.1 ml in triplicate) were taken from both fractions for counting. Residual phenol was removed from the aqueous solution by three ether extractions. The ether was then evaporated from the aqueous phase under a stream of air, and the volume adjusted to 25 ml. After removal of 0.1-ml aliquots for radioactive determination, the entire 25 ml was loaded onto a 2 x 15 cm DEAE-cellulose column which had been equilibrated with 0.01 M Tris, pH 7.2. Chromatography was carried out at 25 C by elution with a 500-ml linear gradient of 0.01 M Tris (pH 7.2) to 0.01 M Tris plus 1 M NaCl. Two-ml fractions were collected and tubes were assayed for absorbance at 260 nm and for radioactivity.

Calculation of H^3 and C^{14} Radioactivity

In order to correct for overlap in counting H^3 and C^{14} simultaneously, the actual H^3 and C^{14} radioactivity were calculated in this manner. Standard solutions of H^3 and C^{14} were prepared (in triplicate) in a toluene-based scintillation fluid and were counted using the H^3 and C^{14} with H^3 isosets. The following values were determined from the counting data:

$$h_1 = H^3 \text{ in } H^3 \text{ isoset} = \frac{\text{net cpm in } H^3 \text{ window}}{H^3\text{-toluene dpm}}$$

$$h_2 = H^3 \text{ in } C^{14} \text{ with } H^3 \text{ isoset} = \frac{\text{net cpm in } C^{14} \text{ with } H^3 \text{ window}}{H^3\text{-toluene dpm}}$$

$$c_1 = C^{14} \text{ in } H^3 \text{ isoset} = \frac{\text{net cpm in } H^3 \text{ window}}{C^{14}\text{-toluene dpm}}$$

$$c_2 = C^{14} \text{ in } C^{14} \text{ with } H^3 \text{ isoset} = \frac{\text{net cpm in } C^{14} \text{ with } H^3 \text{ window}}{C^{14}\text{-toluene dpm}}$$

Since h_1 was equal to 0.002, its value was considered negligible. From the calculations of h_2, c_1 and c_2 above, the actual H^3 and C^{14} dpm in experimental samples were calibrated by the following formulae:

$$C^{14}\text{dpm} = \frac{N_1}{c_2}$$

$$H^3\text{dpm} = \frac{N_2 - (C^{14}\text{dpm} \times c_1)}{h_1}$$

where N_1 = cpm of sample in C^{14} with H^3 isoset

N_2 = cpm of sample in H^3 isoset.

Identity of Material Eluted in 0.7 M NaCl

The greatest amount of ultraviolet absorbing material and radioactivity eluted from the DEAE-cellulose column at 0.01 M Tris and 0.7 M NaCl. The nature of this material was resolved by the subsequent procedures. The fractions under the peak were pooled, lyophilized and redissolved in a smaller volume of water. The samples were dialyzed against water at 5 C for 60 hours with 6 changes. Absorbance at 260 nm and radioactivity were measured before and after dialysis to determine loss of material (i.e., whether the ultraviolet absorbing compound was dialyzable). Aliquots of the retentate were lyophilized and then: 1) analyzed for RNA content, 2) hydrolyzed in 0.5 N NaOH for 18 hours and analyzed for RNA, 3) assayed for DNA content after a hot 5% trichloroacetic acid extraction, 4) used for protein determinations.

Biochemical Analysis of Cultures Treated Up to 16 Hours

Conidia from strain 74A were inoculated into flasks containing minimal media to give 2 x 10^6 conidia/ml. Cultures were incubated on a rotary shaker at 30 C for 6 hours, after which benomyl was added to one-half the number of cultures to give 1 µg/ml. The flasks were incubated further, and a control and treated culture were removed at 1, 2, 4, 8 and 16 hours after addition of benomyl. Mycelia were filtered, washed, lyophilized and weighed.

One hundred mg of each sample (except for the 1 hour samples, which were 85 mg) were homogenized in distilled water in the Omnimixer for 1½ minutes at maximum speed. The container was rinsed several times and volumes brought to 30 ml. The samples were sonicated 20 minutes at 5-15 C until only empty wall fragments were observed microscopically. The cell wall was sedimented by centrifugation at 9,750 x g, and the resulting pellets were washed 5 times with water. The first two supernatants were combined. The pellets (cell wall) and supernatants (soluble fraction) were lyophilized and weighed. The soluble fractions were resuspended in a small volume of distilled water and homogenized with a Thomas glass homogenizer. These samples were brought to a known volume, then diluted for further use. Assays which were performed on each of the soluble fractions included protein, total hexose, total phosphorus, labile and extra-labile phosphorus, DNA and RNA.

Extraction of Nucleosides, Nucleotides and Bases

One gram each of control and treated (1.5 µg/ml for 48 hours) mycelial powder was first extracted for 1 hour using 30 ml of phenol saturated with 0.01 M Tris buffer, pH 7.2. After centrifugation at 27,000 x g, the supernatants were removed. The pellets were washed in 0.01 M Tris, then the aqueous supernatant and wash were pooled. Residual phenol was removed by extracting 3 times with ether. Absorbance of the aqueous solution was measured at 260 nm and 280 nm.

DEAE-cellulose chromatography. The entire sample from control or treated cultures was placed on a 2 x 15 cm DEAE-cellulose column prepared and equilibrated as previously described. Fractions were eluted stepwise at pH 7.2 with 150 ml each of 0.01 M Tris, 0.01 M Tris plus 0.2 M NaCl and 0.01 M Tris plus 1.0 M NaCl. The column was run at room temperature and 2-ml fractions were collected. The absorbance at 260 nm was used to determine the elution profile for the column.

Desalting of nucleotide fractions. The peaks eluting with Tris plus 0.2 M NaCl were presumed to contain nucleotides by comparison with the elution pattern from a similar column run with standard samples. Therefore, fractions under the peaks were pooled, and salt was removed by passage through a 2 x 100 cm Biogel P-2 (polyacrylamide) column at room temperature. The column was eluted with dilute ammonia at pH 8-9 and fractions were collected in 4-5 ml volumes. The A_{260} of the fractions was determined. Samples under the peaks were pooled and evaporated to dryness at approximately 4 C on a Flash evaporator. The samples were redissolved in a small amount of distilled water and transferred to a conical centrifuge tube. They were again evaporated to dryness on an Evapo-mix (Buchler) using a vacuum pump.

Paper chromatography. Evaporated samples of control and treated material were redissolved in 1.0 ml of water and the absorbance at 260 and 280 nm was again recorded. One-half of each sample was re-evaporated, dissolved in 25 µl

of water and applied to sheets of Whatman #1 chromatography paper. Descending chromatography was carried out according to the procedure outlined by Miller (1972) using isobutyric acid:NH_4OH:H_2O (66:1:33) as a solvent. Once the chromatograms were dry, bands of ultraviolet absorbing material were located with a short-range ultraviolet light. The bands were cut out, and washed with ethanol:ether (1:1) to remove the isobutyric acid, which interferes with the ultraviolet spectrum. The papers were then eluted with water using the method described by Miller (1972). After evaporating the eluate to dryness, it was redissolved in 1 ml water; the A_{260} was determined for a quantitative estimate of the amount of nucleotide present.

Identification of nucleotides. Nucleotides were tentatively identified by the distance traveled in isobutyric acid:NH_4OH:H_2O relative to known standards. In addition, the components were rechromatographed in an ascending direction using i-propanol:NH_4SO_4:H_2O (1:40:9) and compared with standards. A more conclusive identification was made by comparison of their ultraviolet spectra with the spectra of known nucleotides. Neutral, acidic and basic spectra of all eluted samples were recorded. The samples (after A_{260} was determined) were diluted (if required) to read no more than 1 absorbance unit per ml at 260 nm. The ultraviolet spectrum of a 1 ml sample was scanned from 320 nm to 220 nm with a Cary 14 Recording Spectrophotometer at a scan speed of 0.5 nm per second and a chart speed of 2 inches per minute.

After the spectrum in water was completed, 10 µl of 4 N HCl (pH 1-2) was added to the cuvette, mixed and the spectrum recorded as before. The basic spectrum was taken after adding 10 µl of 8 N NaOH (approximately pH 12).

Analysis of the number of phosphate groups on the nucleotides was accomplished by electrophoresis. Samples were applied to Whatman 3 MM chromatography paper so that they could be seen under ultraviolet light. They were then subjected to electrophoresis in 0.1 M citrate buffer, pH 3.5, for 1 hour at 500 volts. After drying the paper, ultraviolet absorbing material was identified by comparison with known standards.

Identification of nucleosides and bases. Peaks from control and treated samples which eluted from DEAE-cellulose at 0.01 M Tris were thought to contain a mixture of free bases and nucleosides. The fractions within these peaks were pooled, lyophilized and dissolved in 2 ml of water. After measuring absorbance at 260 nm, samples were applied to Whatman #1 chromatography paper and chromatographed in a descending direction using as solvent isobutyric acid:NH_4OH:H_2O. Ultraviolet absorbing bands were tentatively identified by corresponding migration with standards. Ultraviolet absorbing areas were cut out and eluted with water as previously described. After evaporation and redissolving in water, only the neutral spectra were taken.

Ascospore Segregation

Spores of 74A were streaked onto crossing media containing 0, 0.1, 0.125, 0.15, 0.175 and 0.2 μg/ml benomyl. The slants were incubated at 34 C and then at 25 C to allow the formation of protoperithecia. Then conidia from 74a were added to each tube and cultures were left at room temperature for 2-3 weeks. After ascospore maturation and release, 2 ml of sterile water was added to each culture, mixed thoroughly and then filtered through cheesecloth to give an ascospore suspension.

Ascospores were tested for viability and mutations in this fashion. Aliquots of the spore suspensions formed from each cross were added to 100 ml of sorbose medium plus 0.1% yeast extract and then heat shocked at 60 C for 30 minutes. Plates were poured and incubated for 52 hours at 34 C and then at 25 C to select for temperature sensitive mutants. Colony counts were made from the plates and then, in some instances, conidia were transferred to minimal media to test for mutation to auxotrophy.

In addition to the plate counts, approximately 3300 spores from each cross were counted with a hemocytometer to calculate the number of spores per ml and the ratio of black (viable) to white (non-viable) ascospores. Percent viability was estimated by dividing the number of colonies per ml by the total number of spores per ml.

Mutagenicity of Benomyl

Benomyl treatment of wild-type Neurospora. Fifty ml of minimal agar medium in flasks were kept warm after autoclaving. Benomyl was added to the medium to give 0, 0.23, 0.25, 0.27 and 0.29 µg/ml, and then conidia from 74A were added so that the final concentration was 1 x 10^6/ml. The media was thoroughly mixed, allowed to harden and incubated at 34 C. Conidia from the flasks containing 0, 0.27 and 0.29 µg/ml benomyl were used for filtration enrichment experiments.

Filtration enrichment. Round bottom flasks containing 250 ml minimal media were inoculated with conidia from control and treated cultures to give a suspension of 5 x 10^5 conidia/ml. Cultures were incubated at 25 C on a reciprocal shaker for 8 hours. Germinated conidia were then filtered off by pouring the liquid through 4 layers of cheesecloth into a sterile flask. Cultures were allowed to incubate and were filtered every 3 hours for the first 15 hours, then every 4-5 hours and lastly, every 8 hours or when germinated conidia were observed (Woodward et al., 1954).

At 62, 70, 82 and 88 hours, equal aliquots from each flask were added to warm complete sorbose media and to minimal sorbose media. Pour plates were made and incubated at 34 C. Colonies appearing on minimal were scored as wild type, and those on complete as wild type plus auxotrophic mutants.

Genetic Methods

Isolation of Benomyl-Resistant Mutants

Ultraviolet irradiation. An aqueous suspension of conidia from 5 day-old cultures of hist-1A was filtered through cheesecloth and the concentration was adjusted to approximately 10^7 conidia/ml. A thin layer of the suspension was poured into a sterile petri dish. Conidia were irradiated at 50% kill (125 seconds) under a General Electric G30T8 (30 watt) germicidal lamp at a distance of 6 inches from the light source. Aliquots of treated conidia were suspended in sorbose media containing histidine and either 0, 0.5 or 1.0 µg/ml benomyl. Pour plates were made and were subsequently incubated at 34 C for 4 days. Benomyl-resistant mutants were transferred from plates to slants of minimal media with histidine and 0.5 or 1.0 µg/ml benomyl.

Selection of resistant ascospores. To assure that benomyl-resistant strains were homokaryotic for the mutant allele, all isolated mutants were crossed to 74a. Procedures for crossing and random ascospore selection have been described by Perkins (1959). Ten ascospores were taken from each cross and transferred to slants of minimal media plus histidine and the concentrations of benomyl (0.5 or 1.0 µg/ml) on which resistant conidia were originally selected. Spores were heat shocked at 60 C for 30-40 minutes and incubated at 34 C. Forty-one isolates were recovered.

Histidine auxotrophs among mutants isolated in this manner were determined by transfer to minimal media and scoring for growth. Mating types of the mutants were ascertained by crossing each mutant to both 74A and 74a. These mutants were used in mapping the gene for benomyl resistance.

Mapping Procedures

In order to assign the resistant gene to a linkage group, preliminary crosses between resistant mutants and alcoy strain were performed and scored according to methods outlined by Perkins et al. (1969). Approximately 100 spores were picked from each cross. After scoring for temperature sensitivity, yellow, orange and albino conidia, isolates were transferred to benomyl slants and scored for resistance. Per cent germination was also noted.

Following the cross to alcoy, it was necessary to determine which of two linkage groups contained the benomyl-resistant locus. Consequently, benomyl-resistant mutants were then crossed with strains 1207 and 1208 which carry a tryp-1 marker on linkage group III and a ylo-1 marker on linkage group VI. Approximately 100 ascospores from each cross were isolated. After incubation at 34 C the cultures were scored for tryp$^-$ by fluorescence of media supplemented with indole, and for yellow or orange conidia. Isolates were then transferred to benomyl slants and scored for resistance.

Once the resistant gene had been relegated to a specific linkage group, the position of the locus was determined

relative to three other markers in that linkage group. The resistant mutants were crossed to strains 2091 and 2092 which contain the markers tryp-2, ylo-1 and chol-2. Approximately 120 ascospores were isolated from each cross. Isolates were scored for yellow or orange conidia and then transferred to: 1) minimal media plus choline to test for tryptophan prototrophy, 2) minimal media plus tryptophan to score for choline prototrophy, 3) minimal plus tryptophan, choline and benomyl to determine resistance, 4) minimal plus choline and tryptophan as a control. Phenotypic evaluations were made a great deal easier by using liquid media for all the above tests. Map distances between benomyl and the markers were calculated from the per cent recombination which resulted from these crosses.

Resistance Levels of Mutants

Tolerance levels to different concentrations of benomyl were determined for all those mutants used in mapping studies. Conidia from each mutant were inoculated into Ryan tubes containing media with various concentrations of benomyl. Linear growth at 34 C was measured over a period of 24 to 96 hours. Average growth rates at each concentration were computed from a plot of millimeters of growth vs. time.

Formation of Heterokaryons

From crosses between benomyl-resistant mutants and 2091 or 2092, recombinants were selected which exhibited either resistance or sensitivity and only one auxotrophic phenotype

(i.e., either tryp$^-$ or chol$^-$, but not both). These recombinants were tested for mating type on fluffy^P in the following manner. FluffyA and fluffya were inoculated onto separate plates of minimal medium. After confluent growth was observed, conidia from recombinants were inoculated onto fluffy. Subsequent to incubation at room temperature for 1 week, the appearance of mature perithecia was an indication of a successful cross. The recombinant in question was then noted to be of opposite mating type as the fluffy strain to which it was crossed.

Heterokaryons between recombinants were produced by inoculating onto minimal medium two strains which exhibited opposite phenotypes for benomyl resistance and for tryptophan and choline requirement, but which were of the same mating type. Both cultures used in the formation of heterokaryons were tested on minimal medium to ensure that the tryp and chol mutations were non-leaky.

Heterokaryons were tested for resistance by transfer to benomyl slants. Growth rates and resistance levels were examined for two of the heterokaryons using Ryan tubes containing media with graded levels of benomyl.

RESULTS

Growth Experiments

The initial experiments in this investigation were designed to study the effects of benomyl on germination and growth of N. crassa under different conditions so that the information obtained might serve as a basis for subsequent experiments. The results presented in Figure 3 illustrate how various concentrations of benomyl affect growth of Neurospora wild type strain 74A on solid medium. Benomyl at 0.05 µg/ml caused a stimulation of growth, but an increase to only 0.10 µg/ml produced 16% inhibition after 96 hours. Approximately 54% inhibition occurred at 0.175 µg/ml and no increase in growth was observed at concentrations of 0.25 and 0.30 µg/ml. Although it is not shown here, benomyl had a similar effect on growth of SF26, a strain exhibiting hyperproduction of amylase and invertase.

In liquid cultures (Figure 4), the addition of benomyl at the time of inoculation did not prevent an increase in absorbance (Klett units), indicating that germination of conidia is not inhibited and that some growth continues in the presence of the toxicant. By this procedure inhibition was not observable for 2-3 hours, but the degree of inhibition became progressively larger with time. Maximum inhibition occurred at approximately 1.0 µg/ml; by 10 hours the absorbance was only one-half that of the control culture.

Figure 3. The effect of various concentrations of benomyl on growth of N. crassa 74A on solid medium. Benomyl was added to the medium prior to inoculation. Symbols: Control (●) and benomyl treated at 0.05 (○), 0.10 (■), 0.125 (□), 0.15 (▲), 0.175 (△), 0.20 (◐), 0.25 (◑) and 0.30 (◧) µg/ml.

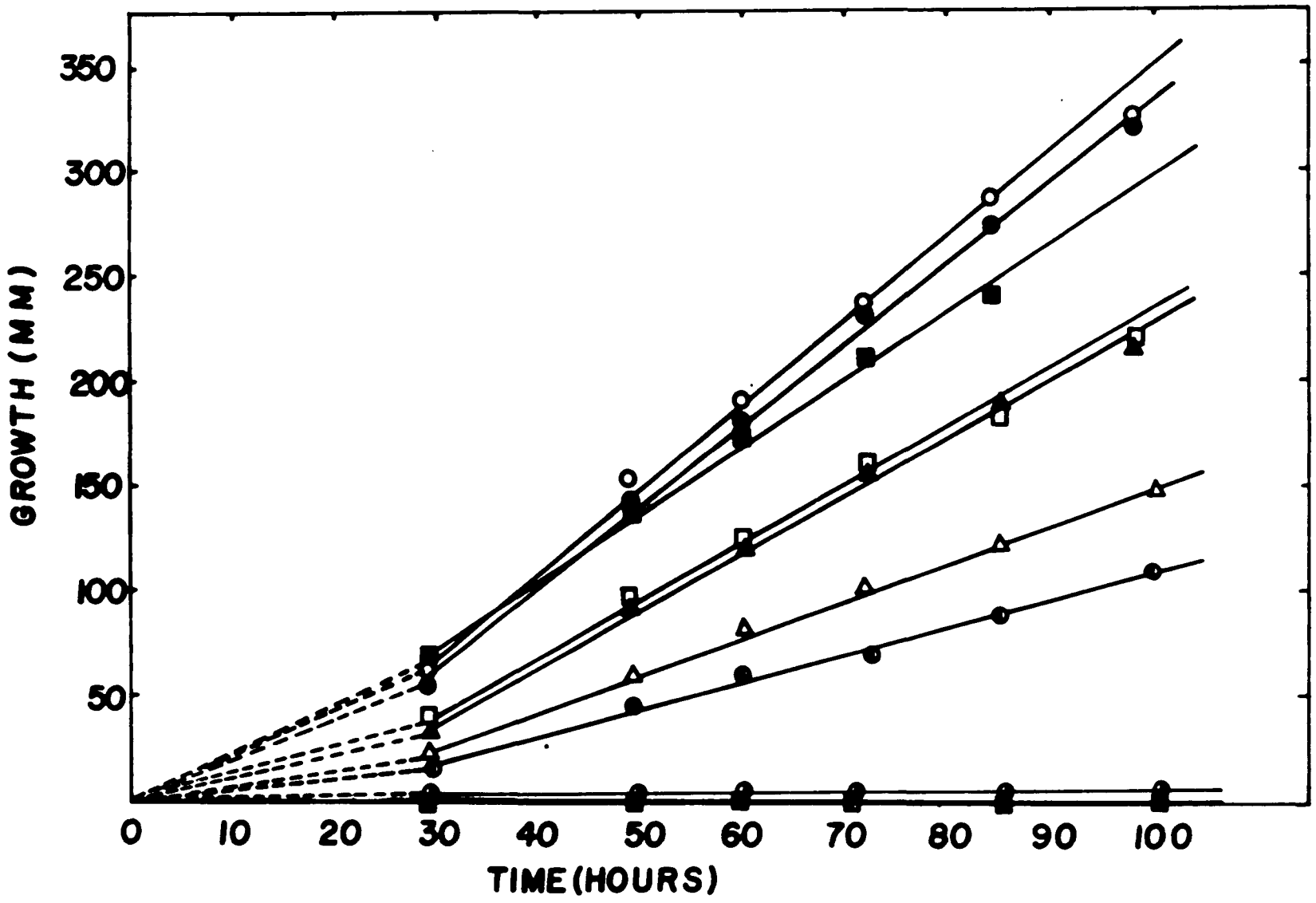
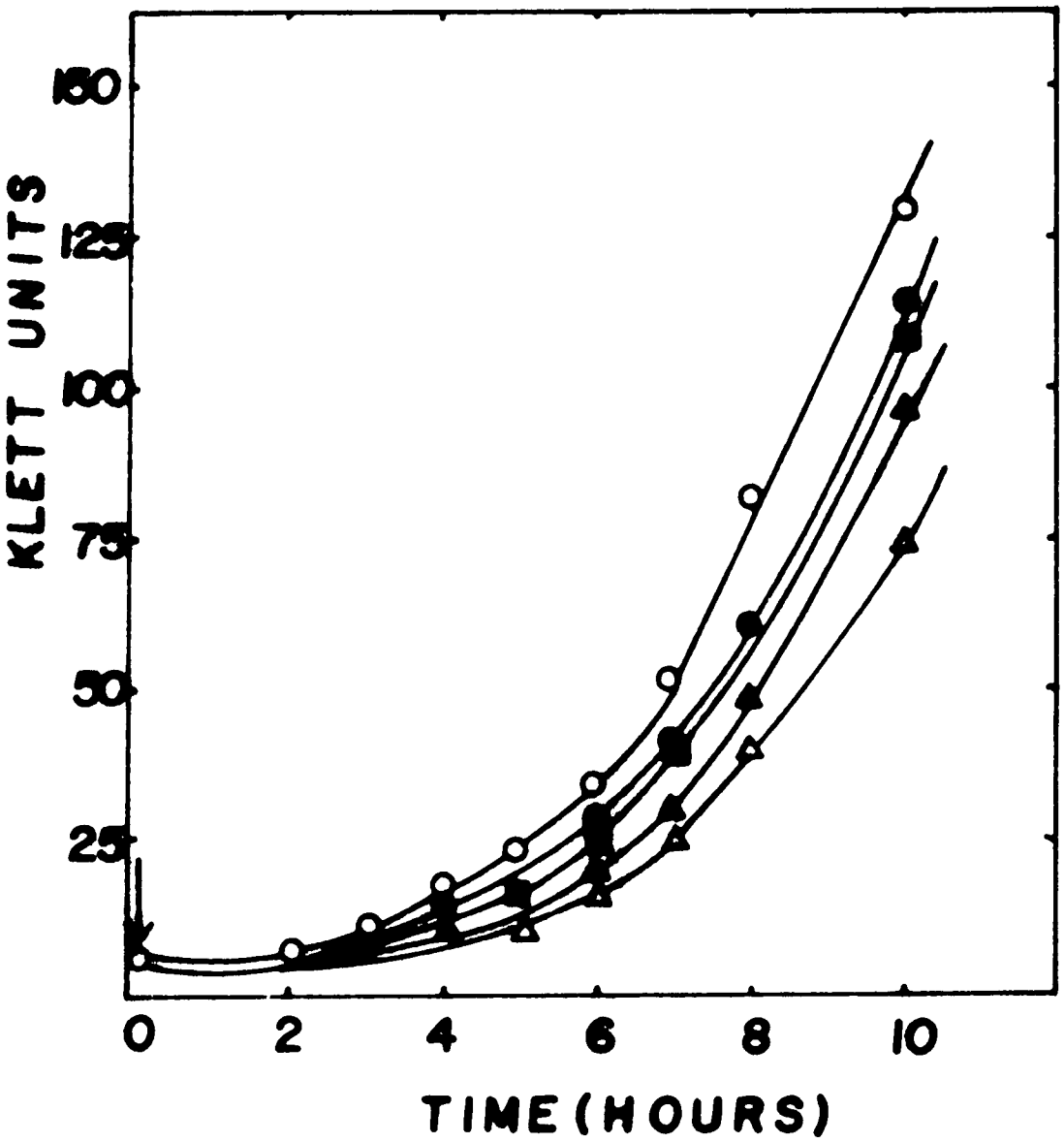

GROWTH (MM)
350
300
250
200
150
100
50
0
10
20
30
40
50
60
70
80
90
100
TIME (HOURS)

Figure 4. The effect of various concentrations of benomyl on growth of N. crassa SF26 in liquid culture. Benomyl was added at the time of inoculation. Symbols: Control (●) and benomyl treated at 0.1 (○), 0.15 (□), 0.2 (▲) and 0.25 (△) µg/ml.


KLETT UNITS
150
125
100
75
50
25
0
2
4
6
8
10
TIME (HOURS)

Very low levels of benomyl (0.1 μg/ml) again had a slight stimulatory effect on growth.

When benomyl was added to pregerminated cultures of Neurospora (Figure 5), a comparable pattern developed such that no reduction in growth was revealed until 2-3 hours after introduction of the fungicide and a substantial difference was seen only after some 4-5 hours. Here again, a gradual increase in absorbance was noticed in all treated cultures.

Figure 6 demonstrates the effect of benomyl on growth of 74A as determined by changes in lyophilized dry weight of mycelia. Treated cultures continued to gain in weight for 4 hours, just as did control cultures. After this period the extent of inhibition increased gradually with time. At 16 hours, however, inhibition calculated by this procedure was only 38% whereas inhibition measured by decrease in absorbance units was 50%.

The growth of slime was affected to a much greater extent by the addition of benomyl to the medium (Figure 7). Only a slight increase in absorbance occurred initially, and then growth halted entirely. This effect is probably due to a lack of cell wall in slime and more rapid permeation of benomyl into the cell. At later stages of incubation, absorbance decreased somewhat, probably indicating lysis and premature cell death.

Figure 5. The effect of various concentrations of benomyl on growth of N. crassa SF26 in liquid culture. Benomyl was added after 4 hours preincubation of conidia. Symbols: Control, (●), and benomyl treated at 0.1 (○), 0.2 (■), 0.4 (▲), 1.5 (△) µg/ml.

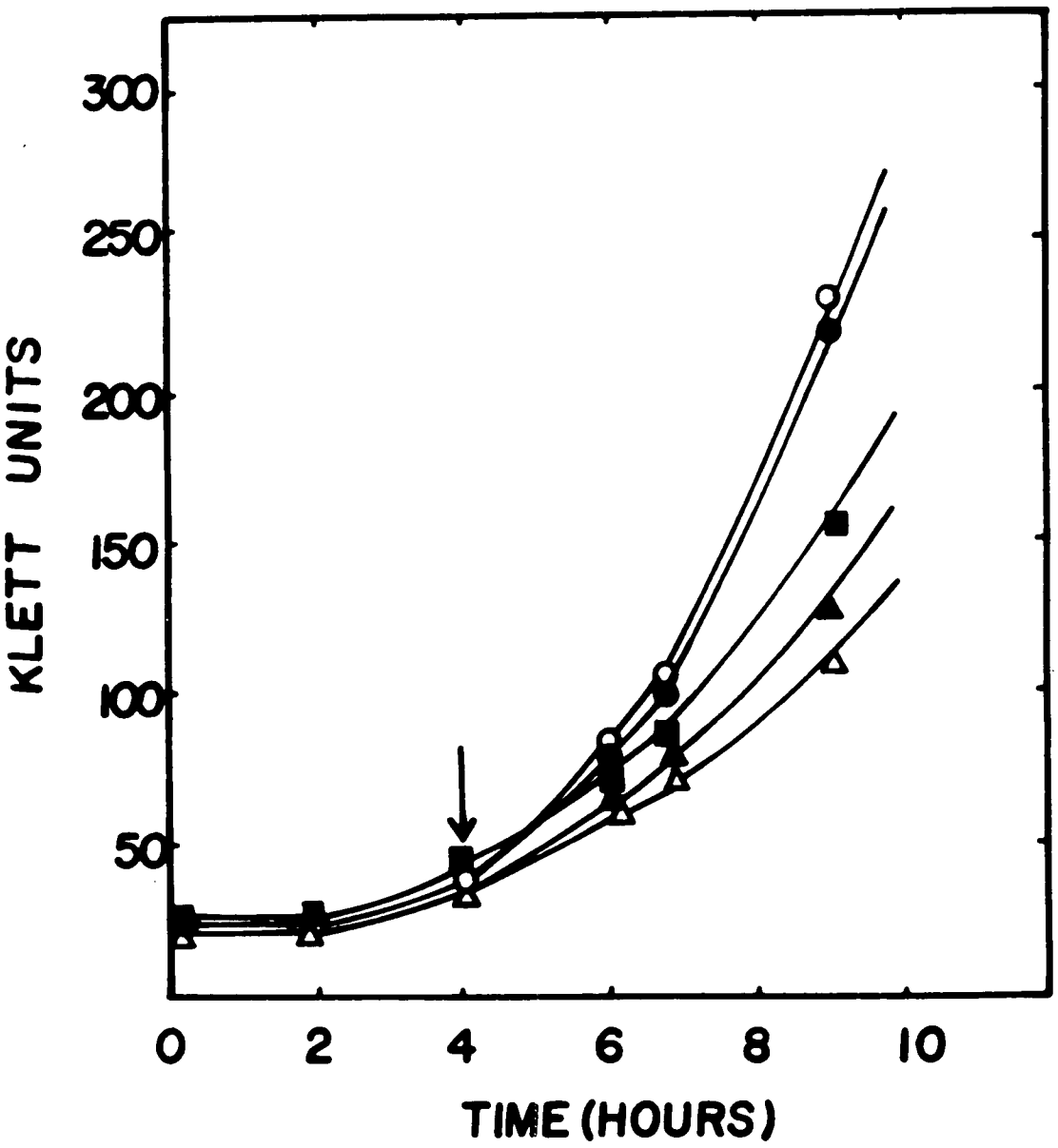

KLETT UNITS
300
250
200
150
100
50
0
2
4
6
8
10
TIME (HOURS)

Figure 6. The effect of 1.0 μg/ml benomyl on growth of N. crassa 74A as measured by lyophilized dry weight of filtered cultures. Benomyl was added after 6 hours preincubation and cultures were removed at 1,2,4, and 16 hours after addition of the fungicide. Symbols: Control (●), benomyl treated (○).

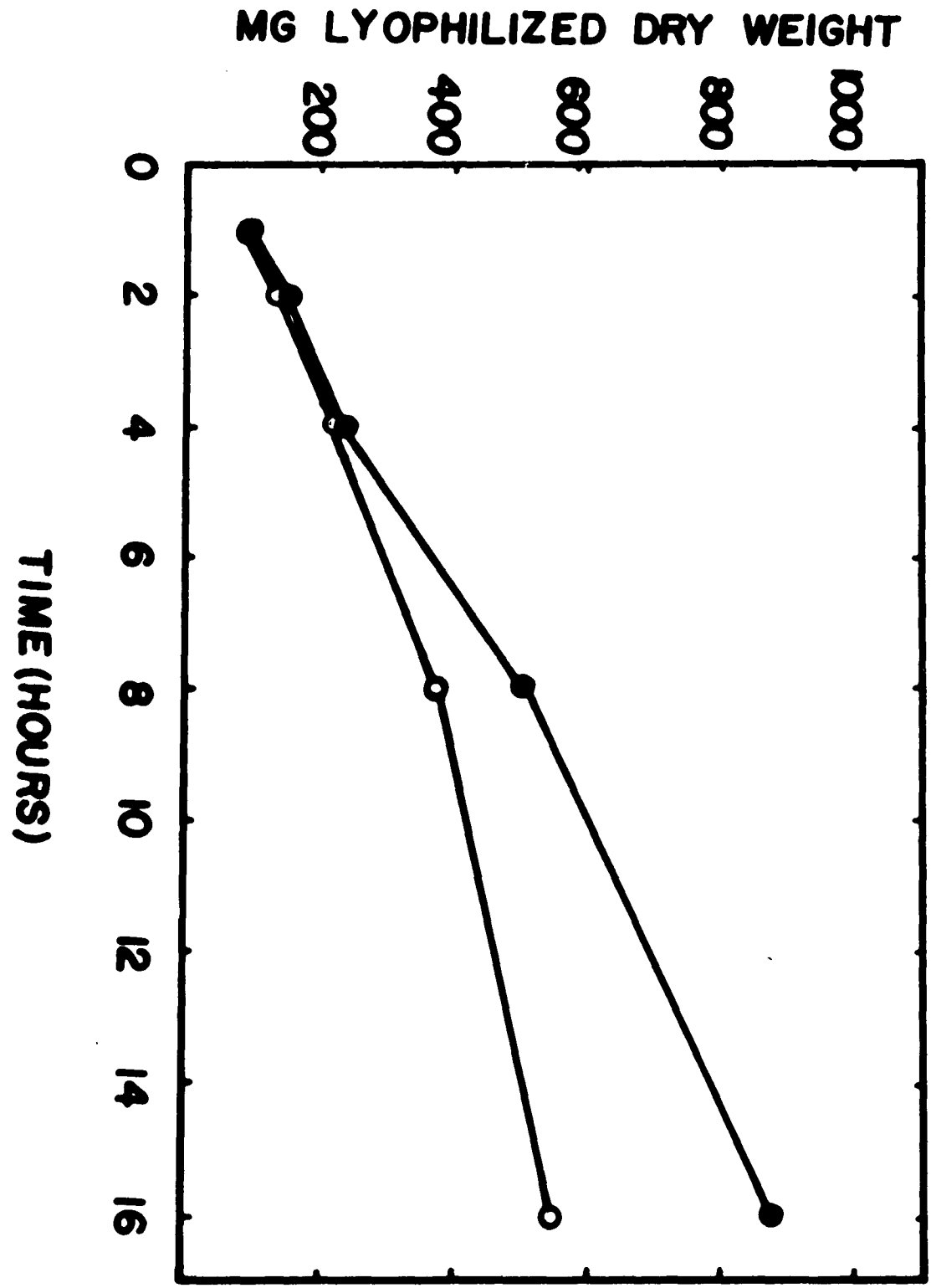
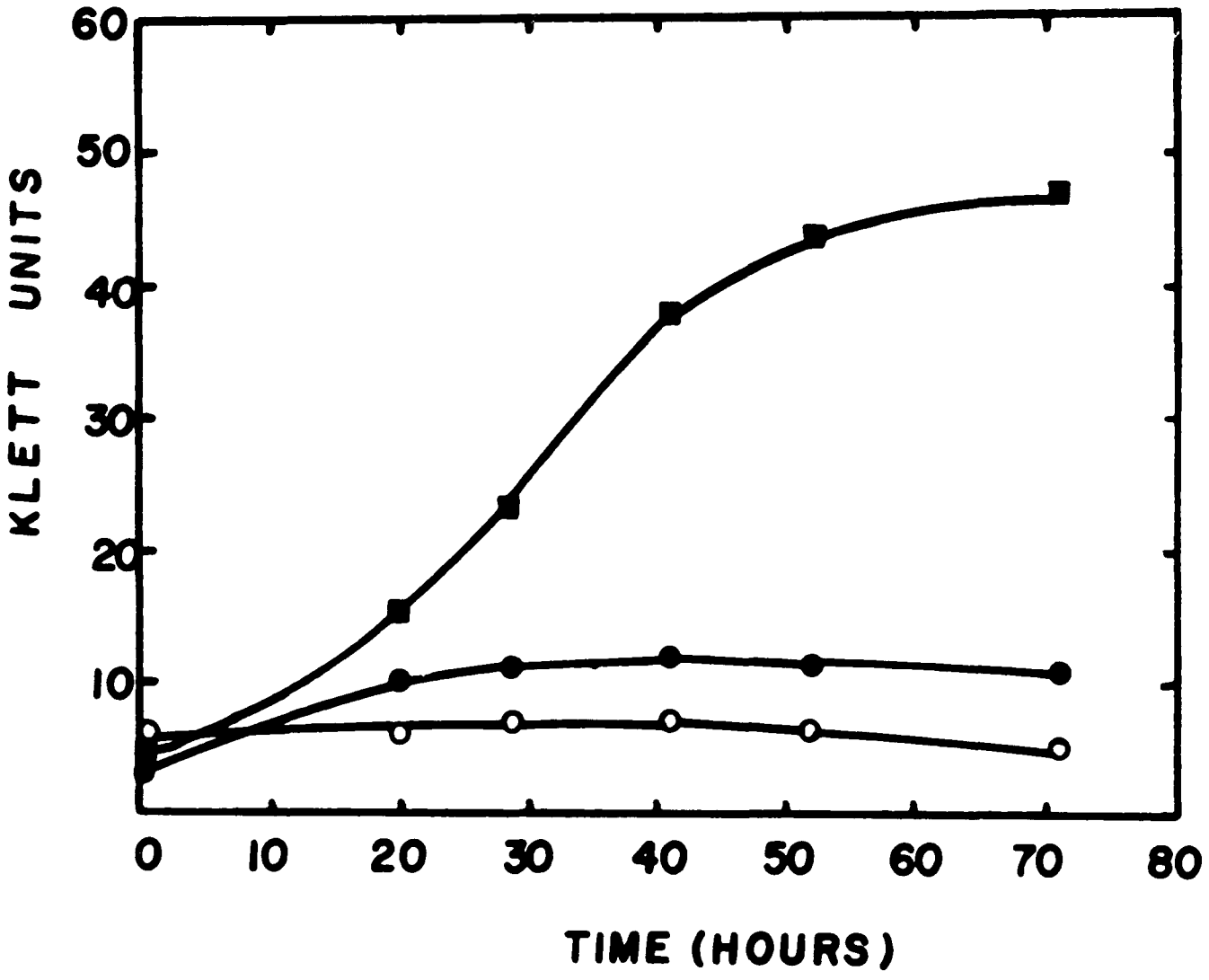

MG LYOPHILIZED DRY WEIGHT
200
400
600
800
1000
0
2
4
6
8
10
12
14
16
TIME (HOURS)

Figure 7. Benomyl induced inhibition of slime. Benomyl was added at the time of inoculation. Symbols: Control (■) and benomyl treated at 0.5 (●) and 1.5 (○) µg/ml.


KLETT UNITS
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
TIME (HOURS)

Effects of Benomyl on Morphology and Ultrastructure

The inferences drawn from growth studies are that benomyl does not affect germination of conidia and that the consequences of treatment are not observed until after a lag period of 2-4 hours. To substantiate these deductions, photomicrographs of cultures containing 1 μg/ml benomyl were made at intervals following inoculation. At 1 hour (Figure 8, A and B) there was very little difference in appearance of conidia from control and treated cultures. By 2 hours (Figure 8, C and D), however, nearly all the normal conidia had produced germ tubes, some as long as 45 μ, and measuring 3-6 μ in width. Treated conidia were beginning to germinate, but displayed foreshortened, very broad germ tubes, often with multiple branching. At 4 hours (Figure 9, A and B), the differences between control and benomyl treated cells became quite obvious. Germ tubes of normal Neurospora were long, 3 μ wide and contained distinct cross walls. Large vacuoles were present in many of the conidia. By this time nearly all the treated conidia had germinated, and Figure 9, B shows the characteristic distortion of germ tubes and the granular appearance of the cytoplasm in benomyl-grown cells. These characteristics are also shown in Figure 9, D, which was taken at 6 hours. In the 6 hour cultures, multiple germ tubes emanating from a single conidium and numerous vacuoles were apparent. Conidia were swollen to as much as 12-18 μ in diameter, whereas untreated conidia remained 9 μ in diameter. Hyphae from control cultures were quite elongated.

Figure 8. Photomicrographs of N. crassa 74A treated with 1.0 µg/ml benomyl which was added at the time of inoculation. A, control at 1 hour; B, benomyl treated at 1 hour; C, control at 2 hours; D, benomyl treated at 2 hours. All photographs have equal magnification values and bar on A represents 10 µm.

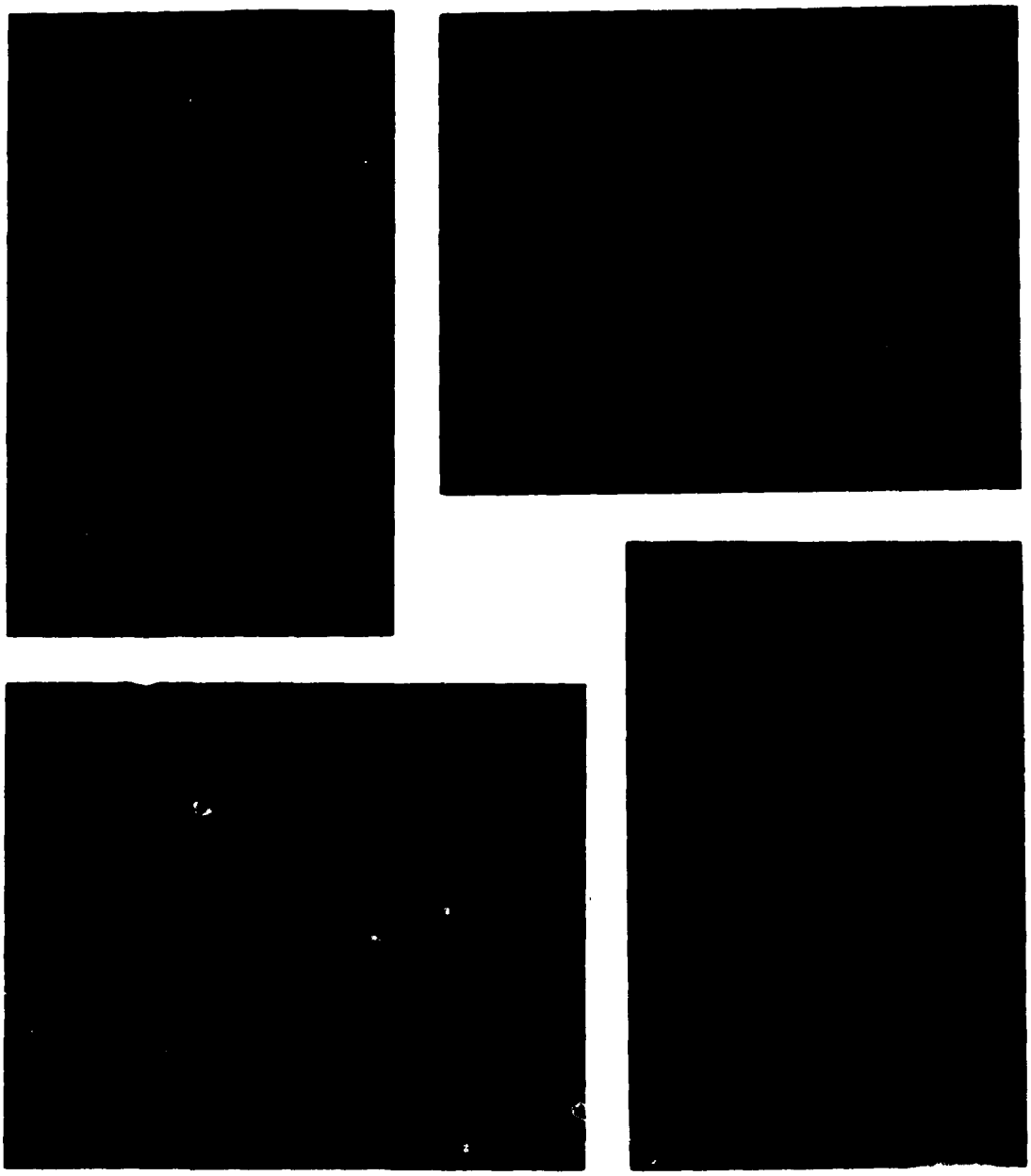


Figure 9. Photomicrographs of N. crassa 74A treated with 1.0 µg/ml benomyl. A continuation of the experiment described in Figure 8. A, control at 4 hours; B, benomyl treated at 4 hours; C, control at 6 hours; D, benomyl treated at 6 hours. All photographs have equal magnification values and bar on A represents 10 µm.

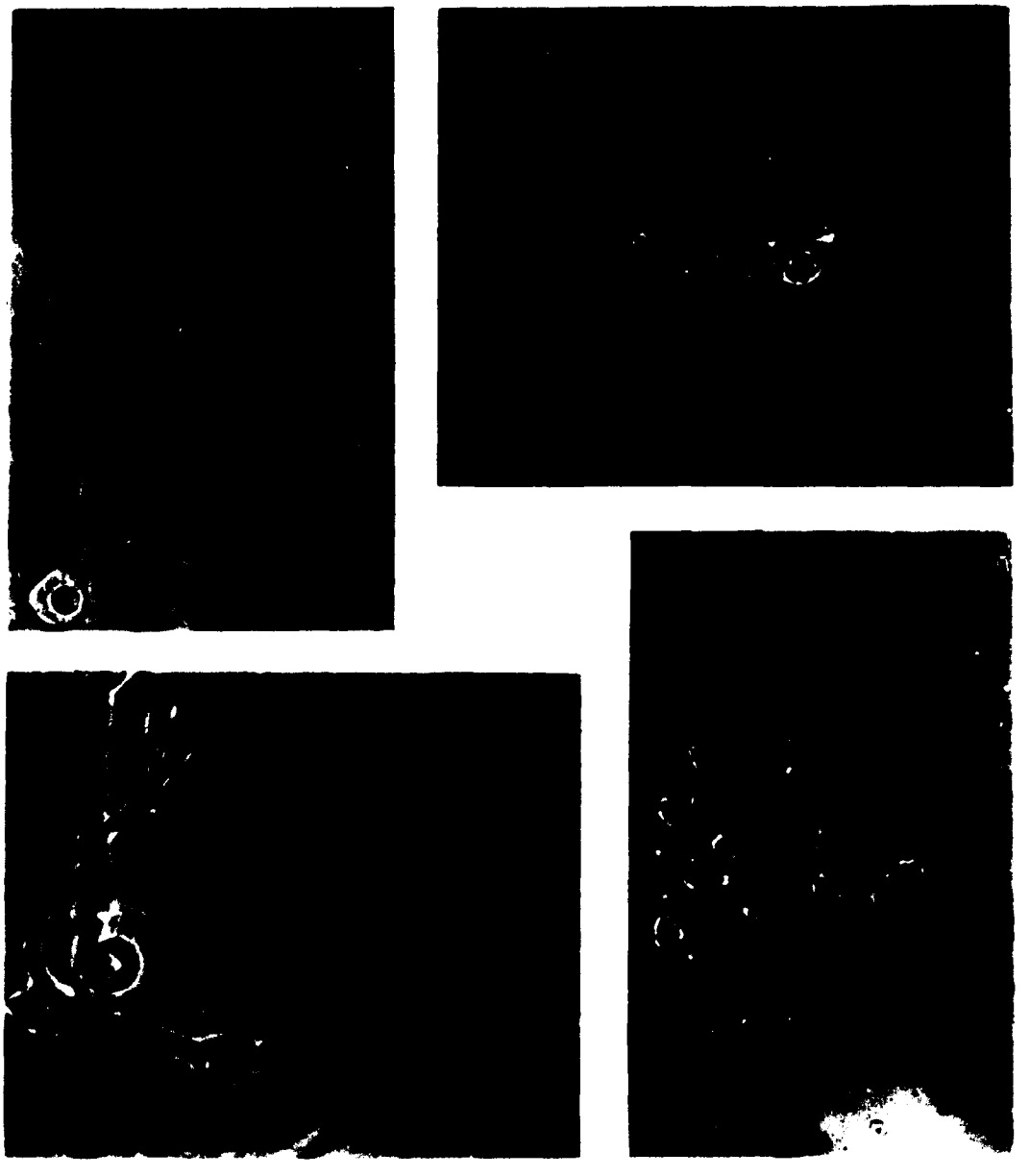
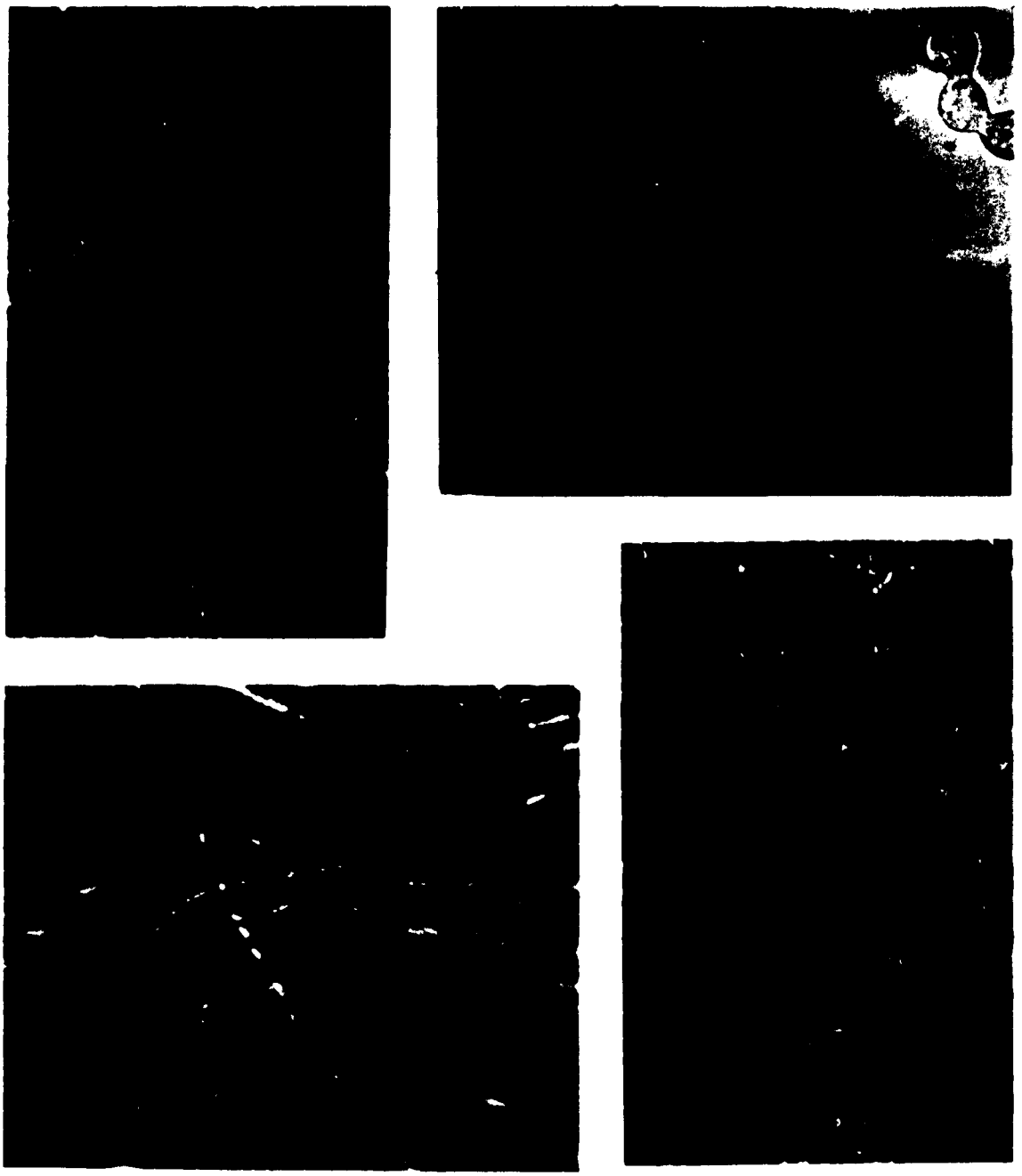


Figure 10. Photomicrographs of N. crassa 74A treated with 1.0 µg/ml benomyl. A continuation of the experiment described in Figure 8. A, control at 8 hours; B, benomyl treated at 8 hours; C, control at 12 hours; D, benomyl treated at 12 hours. All photographs have equal magnification values and bar on A represents 10 µm.



At 8 (Figure 10, A and B) and 12 (Figure 10, C and D) hours, untreated hyphae were further elongated containing well-defined cross walls and vacuoles. Treated conidia, however, continued to produce aberrant hyphae. At the point of cross wall formation, the hyphae were pinched together and cells ballooned outward on both sides, with diameters of 8-12 μ. Hyphal extension appeared to continue at a slow rate in the presence of benomyl.

In order to further elucidate the physical alterations of Neurospora attributable to benomyl, liquid cultures of SF26 were grown in the presence of various concentrations of benomyl, harvested and prepared for electron microscopy. Thin sections of hyphae were observed for any differences in structure when compared with untreated cells. Figures 11, 12A and 12B show electron micrographs of thin sections of normal N. crassa. Hyphae measure approximately 3-4 μ in diameter. The cell wall (0.15 μ thick) is composed of a light and an inner dense layer (Figure 11). Outside the wall is a network of dense fibers which accumulate at the region of cell wall invagination (Figure 12A). Septa are formed by invagination and fusion of the wall with the production of a pore to allow cytoplasmic streaming. These pores may become plugged (Figure 12B) as a result of age or cell damage (Shatkin and Tatum, 1959). Cross walls appear as a light area bound on either side by electron dense regions.

Inside the cell wall is a convoluted plasma membrane surrounding a densely packed cytoplasmic matrix. Associated

with the membrane are coiled membrane bodies also described by Lowry and Sussman (1968), who postulated association with endoplasmic reticulum, and by Van Winkle (1969), who linked them with mitochondrial membranes. Within the cytoplasm appear numerous elongated mitochondria (Figure 11) measuring 0.2-0.3 μ in diameter and flattened vesicles of endoplasmic reticulum. Ribosomes are darkly stained and are suspended throughout the cytoplasm. Large vacuoles are frequently present, some which contain inclusions. Other small vesicles are evident (Figure 12), and it has been suggested by Shatkin and Tatum (1959) that they are lipid granules. These small vesicles are commonly seen near the hyphal tip (Grove and Bracker, 1970) and may serve a function in hyphal elongation. Glycogen (Figure 11) may be identified as irregular aggregates which stain lighter than the cytoplasm (Zalokar, 1961), and zymogen inclusions are seen as large, electron dense hexagonal structures (Figures 11 and 12B). Nuclei (Figure 12A) are difficult to discern with OsO_4 staining, but may be faintly visible as large bodies 1.5-2 μ in diameter surrounded by a double membrane and having irregularly stained regions within.

Electron micrographs of Neurospora treated with benomyl (Figures 13, 14 and 15) demonstrate that the fungicide does cause distortion of hyphal structure and disorientation of the cytoplasm. The cell wall becomes as wide as 0.25-0.50 μ, and in some instances there appears to be a double layer of wall, each layer having a light and dense region with a very

dark area sandwiched between (Figure 13). The outer network of fibrils is considerably reduced (Figure 13) when compared with control cells. Often, cross walls are not normal but are seen as thick, ill-defined areas caused by or resulting in constriction of the hypha at this region (Figure 15). The plasma membrane is not noticeably affected. Mitochondria are not disrupted even at high concentrations of benomyl; however, they often stain with interior electron density. Some sections show a vast number of mitochondria within the hypha (Figure 14). Examination of nuclei (Figure 15) does not indicate that benomyl has detrimental consequences on the ultrastructure of these bodies. Endoplasmic reticulum does not appear to be affected except in degenerating cells. Most evident, however, is the increase in number of vacuoles, which sometimes stain with more density than normal vacuoles. Also seen are an extraordinarily large number of small lipid granules which collect in areas near the plasma membrane.

An examination of slime cultures treated with benomyl for 12 hours did reveal that many cells contained vacuoles which were 4 to 6 times the size of those in untreated cells. Nevertheless, these cultures were 84 hours old, and large vacuoles were sometimes seen in control cells. Therefore, the appearance of large vacuoles may imply incipient cell death rather than a reaction to benomyl. No other components of slime cells, i.e., endoplasmic reticulum, mitochondria, nuclei or plasma membrane, were structurally affected by benomyl treatment.

Figure 11. Thin section of a hypha from normal N. crassa.

Explanation of plates. In the succeeding figures CW signifies cell wall; PM, plasma membrane; ER, endoplasmic reticulum; S, septum; V, vacuole; MB, membrane body; Ve, vesicles; G, glycogen; Z, zymogen inclusions; N, nucleus; SPP, septal pore plug. Bar represents 1 μm.

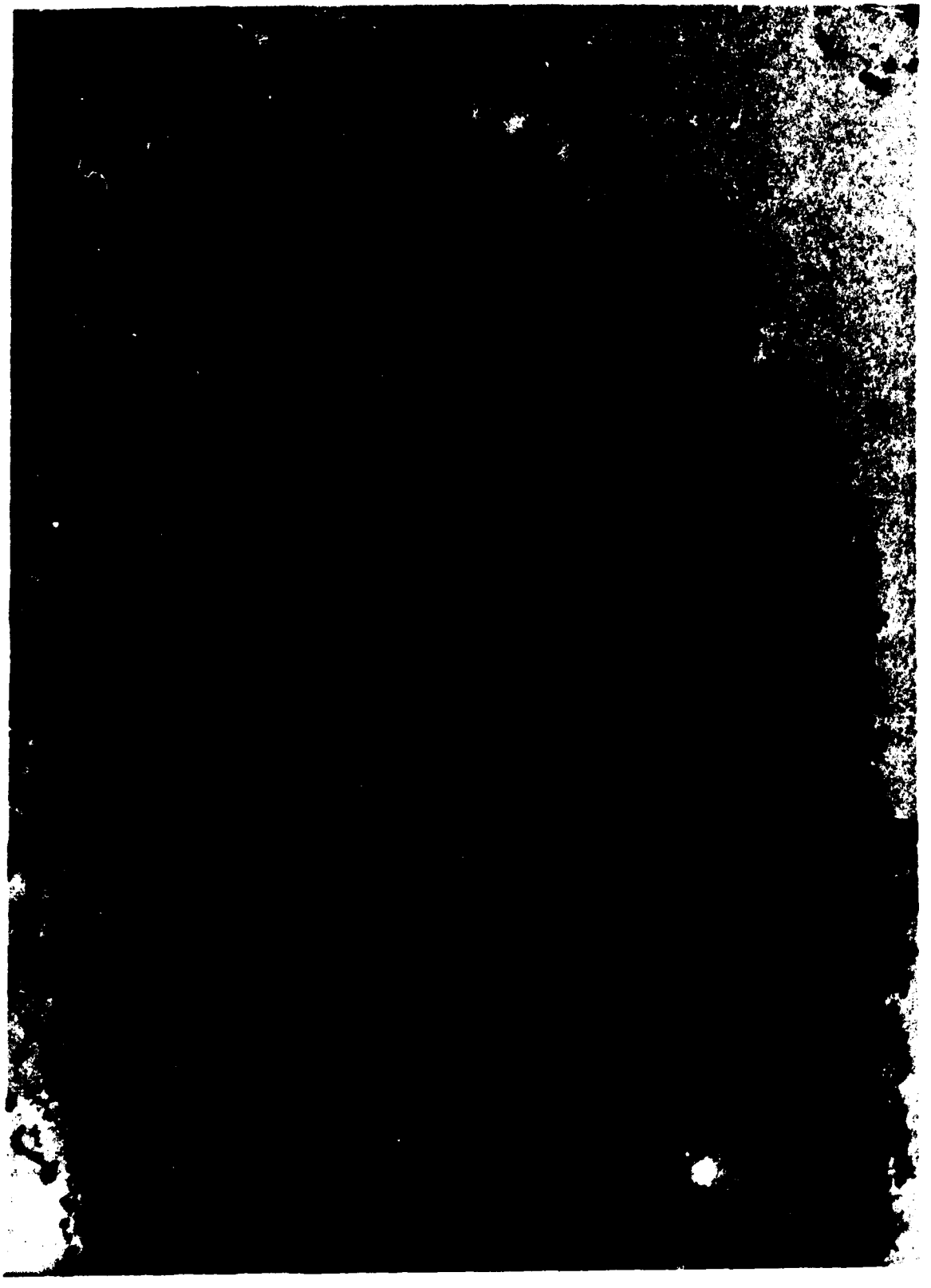


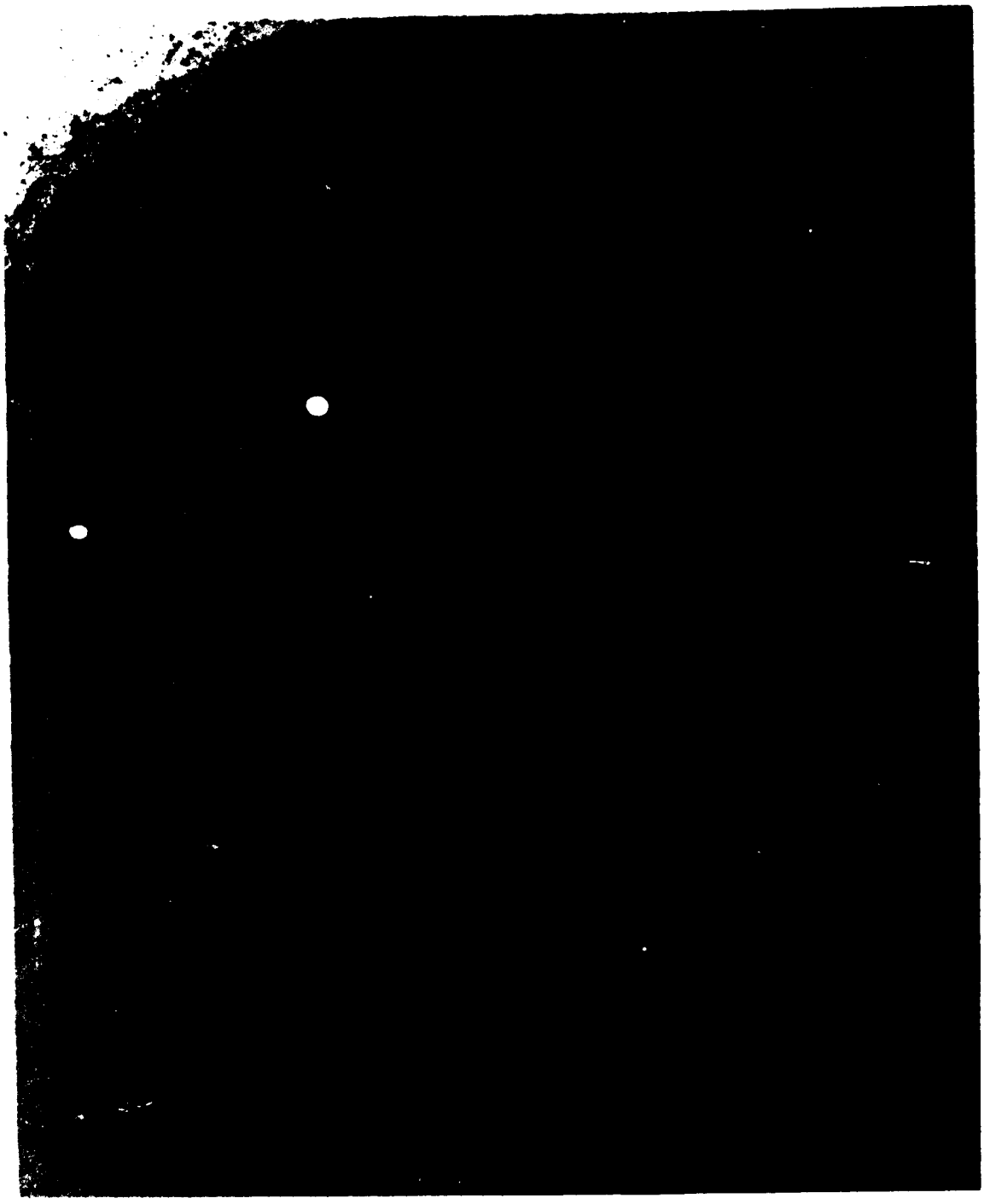
Figure 12. Thin sections of normal N. crassa hyphae showing: A, septum formation, and B, septum from aging cells containing a septal pore plug. Bars represent 1 μm.

Figure 13. Thin section of a hypha from a culture of N. crassa treated with 0.5 µg/ml benomyl for 24 hours. Bar represents 1 µm.

Figure 14. Thin section of a hypha from a culture of N. crassa treated with 0.2 µg/ml benomyl for 24 hours. Bar represents 1 µm.


M

Figure 15. Thin section of a hypha from a culture of N. crassa treated with 0.5 µg/ml benomyl for 24 hours. Bar represents 1 µm.



Effects on Membrane Permeability

That benomyl might damage the selective permeability of the plasma membrane was considered as a possible factor in the toxicity of the compound. However, a comparison of the A_{260} and A_{280} values of the medium from control and treated cultures of wild type and slime indicated there was no leakage of cellular constituents into the external medium. There was also no change in the amount of phosphorus present in the medium when wild type Neurospora was grown in benomyl for up to 24 hours.

Cell Wall Synthesis

Cytological observations of treated hyphae had shown a considerable increase in the thickness of cell walls and had indicated there was some effect of benomyl on cross wall formation. There was also a possibility that benomyl might alter the assembly of cell wall components which could result in a structurally weak wall and would explain the bulging malformations of treated mycelia. Consequently, cell wall material was extracted from mycelial powder of control and treated cultures and then fractionated and analyzed to determine any differences in amounts or in biochemical composition of each fraction. The results of these experiments are summarized in Table 2. Following dialysis and lyophilization, the weight of the sodium dodecyl sulfate-soluble (cytoplasmic) fraction of the control sample was 2.1 times the treated. However, the lyophilized dry weight of the cell

Table 2. Biochemical data on mycelia, cell wall and fractions of cell wall from control and benomyl treated cultures of N. crassa SF26. C, control; T, treated.

Substance determined	Contents of fraction in µg/mg lyophilized dry weight													
	Whole mycelia		Cell wall		Soluble		I		II		III		IV	
	C	T	C	T	C	T	C	T	C	T	C	T	C	T
Lyophilized dry weight	1.5*	1.5*	0.40*	0.70*	1.16*	0.56*	0.06*	0.15*			0.08*	0.13*	0.03*	0.07*
Protein	430	170			600	380	20	10	0	0			20	5
Protein**							100	50						
Total hexose	230	460	670	890	90	49	530	700	250	220	860	830	50	54
Total hexose**							700	820			790	890		
Glucose	0	100			0	25	0	0	35	15	0	0		
Glucose**							110	240			160	210		
Hexosamine									6	6			26	19
Hexosamine**							59	12			11	11	630	500
Acetyl hexosamine***													41	26

*Units in mg

**After acid hydrolysis

***After chitinase digestion

Fraction I. glucosan, galactosamine polymer, peptides, glucuronate

Fraction II. glucose, minor sugars

Fraction III. β-1,3-glucan

Fraction IV. chitin

wall fraction in the control was only 0.4 that of the benomyl treated cell wall fraction. The increase in cell wall of treated mycelia is reflected in each fraction where the dry weights are double those of the controls. However, the ratio of each fraction in dry weight to the dry weight of cell wall (starting material) was the same in every case except fraction III. In this instance the control was 26% and treated was 18% of total cell wall material. These figures indicate that fraction III, which is the β-1,3-glucan described by Mahadevan and Tatum (1965), is affected.

There was a definite decrease in the amount of protein in treated mycelia, and this is most clearly reflected in the cytoplasmic fractions. Total hexose analysis showed an accumulation of these sugars as a result of treatment and upon fractionation nearly all the hexose remained in the cell wall, primarily in fractions I and III. The results agree with those of Mahadevan and Tatum (1965) who reported that 50% of fraction I is glucose and that fraction III is nearly all β-1,3-glucan. There was significantly more hexose in fraction III of the treated sample (1.3 times that of control). This fraction should contain a glucosan and a galactosamine polymer, which (according to analysis of glucose and hexosamine after hydrolysis) are increased and decreased, respectively, by benomyl. Some increase in free glucose was observed in the treated mycelial fraction; however, it is difficult to explain the absence of glucose in the control sample.

The composition of fraction II remains relatively unchanged. Assays of hexosamine and acetyl hexosamine suggest a slight alteration in synthesis of chitin (fraction IV), although differences here may be due to contamination of the fraction by the galactosamine polymer. The values of acetyl hexosamine obtained after chitinase digestion are extremely low (Mahadevan and Tatum, 1965) and we suspect incomplete hydrolysis.

Samples of whole cell wall preparation were analyzed for neutral sugars by gas chromatography (courtesy of the late Dr. R. J. Winzler). The results (Table 3) indicate benomyl treated wall material contains slightly less galactose and more glucose than the control. The total amount of carbohydrate/mg cell wall in treated material was 1.2 times that of the control. The values for total carbohydrate were substantially lower than those obtained by the phenol-sulfuric acid method, although the ratios are similar. Gas chromatographic procedures, however, do not account for the presence of such sugars as glucuronate and amino-sugars, both of which constitute a fairly large proportion of the cell wall.

Table 3. Analysis of neutral sugars from whole cell wall preparations by gas chromatography.

Sugar	μg sugar/mg lyophilized dry weight Control	Treated	Treated / Control
Ribose	1.2	-	-
Mannose	44.1	50.1	1.1
Galactose	45.9	38.7	0.8
Glucose	210.6	276.8	1.3
Total	301.8	365.6	1.2

- Below limit of detection

Amino Acid Analysis

From the preceding work with cell wall fractionation, it became apparent that upon long exposure of cells to benomyl (48 hours), protein synthesis was reduced about 50% in the cell wall as well as the cytoplasm. To determine if the decrease in protein synthesis was due to a block in synthesis of particular amino acids, either for the wall or for the cytoplasmic constituents, a series of amino acid analyses was performed. In the treated mycelia (Table 4), nearly all amino acids were approximately 40% of the control, which would be expected if protein synthesis itself were inhibited 60%. Protein estimated by the Lowry method (Table 2) of the mycelial fraction substantiates this assumption. In treated cells, only arginine and glutamic acid are present in amounts greater than 40% of control. Although the synthesis of these

two amino acids is linked, the significance (if any) of the increase is unclear. Analysis of whole cell wall (Table 5) demonstrated that the levels of most amino acids in treated material were 40-60% of control. Although the Lowry was not performed on cell wall material, the results from fraction I (which contains most of the amino acids of the cell wall) indicate protein is 50% reduced by benomyl. Histidine was markedly decreased and several amino acids (lysine, threonine and proline) existed in greater relative concentrations in treated cell wall than in control.

It is significant that glucosamine (as determined by amino acid analysis) was substantially higher in mycelia of treated cultures (Table 4), and galactosamine was lower in treated wall material (Table 5). The ratio of glucosamine in the treated wall to that of the control (0.74) agrees well with the ratios found by colorimetric estimations of the chitin (fraction IV). The ratio of hexosamine in the treated wall to that in the control wall was 0.73; the same ratio for hexosamine after hydrolysis was 0.78 (Table 2). Determination of hexosamine in fraction I (Table 2) which should contain galactosamine (Mahadevan and Tatum, 1965) gave a ratio of 0.20 (benomyl treated/control) relative to that of 0.35 by amino acid analysis. From the results described above, it seems that benomyl induces an accumulation of glucosamine in the cytoplasm, a point which will become pertinent in light of further data.

Table 4. Amino acid analysis of mycelia from control and treated cultures. Treated cells were grown in the presence of benomyl (1.5 µg/ml) for 48 hours.

Amino acid	umoles amino acid per mg lyophilized dry weight		Benomyl treated / Control
	Control	Treated	
Lysine	.351	.106	.30
Histidine	.048	.024	.50
Ammonia	.543	.354	.65
Arginine	.141	.099	.70
Aspartic acid	.252	.108	.43
Threonine	.149	.081	.54
Serine	.159	.083	.52
Glutamic acid	.326	.202	.62
Proline	.139	.057	.41
Glycine	.248	.089	.36
Alanine	.307	.101	.33
½ Cystine	.026	.007	.26
Valine	.159	.064	.40
Methionine	.042	.015	.35
Isoleucine	.118	.047	.40
Leucine	.205	.078	.38
Tyrosine	.074	.031	.42
Phenylalanine	.092	.038	.42
Glucosamine	.051	.095	1.86
Galactosamine	.047	-	-

Table 5. Amino acid analysis of cell wall material from control and treated cultures. Treated cells were grown in the presence of benomyl (1.5 μg/ml) for 48 hours.

Amino acid	umoles amino acid per mg lyophilized dry weight		Benomyl treated / Control
	Control	Treated	
Lysine	.069	.049	.71
Histidine	.085	-	-
Ammonia	.707	.330	.47
Arginine	.007	-	-
Aspartic acid	.057	.029	.51
Threonine	.051	.041	.80
Serine	.068	.042	.61
Glutamic acid	.040	.024	.60
Proline	.030	.029	.97
Glycine	.050	.021	.41
Alanine	.057	.036	.63
½ Cystine	-	-	-
Valine	.038	.024	.63
Methionine	.003	.002	.67
Isoleucine	.024	.012	.50
Leucine	.030	.014	.47
Tyrosine	-	.011	-
Phenylalanine	-	.011	-
Hydroxyproline	trace	trace	-
Glucosamine	.428	.315	.74
Galactosamine	.173	.060	.35

Invertase Production

From the preceding experiments it appeared that benomyl had produced modifications in the composition, and perhaps the structure, of the cell wall. It was conceivable, therefore, that the effects of benomyl on growth might be due to some benomyl-induced malfunction of the wall and/or the associated plasma membrane. Since invertase is an enzyme associated with the cell wall and is also secreted into the medium, it seemed logical to investigate invertase activity as a parameter of cell-surface functions. There was also a possibility that interference with the activity or synthesis of this enzyme would result in a scarcity of utilizable carbon source for the organism. The results of invertase assays on 24-hour cultures (Table 6) verify that indeed invertase activity is markedly decreased. There was no difference when benomyl was added at the time of inoculation or after 6 hours of growth.

To determine whether inactivation of invertase was the cause of growth inhibition, strain 74A was grown in minimal media containing 1.5% glucose and various concentrations of benomyl. The results showed that Neurospora was slightly less sensitive to benomyl, but inhibition still occurred. After 10 hours of growth in benomyl, there was no inhibition observed at 0.15 and 0.2 µg/ml of the toxicant. Inhibition at 0.25 µg/ml was 30% and at 0.5 µg/ml growth was suppressed by 40%.

Table 6. Invertase activity of control and benomyl treated mycelia from 24-hour cultures of N. crassa SF26.

Sample	Glucose (mg/ml)	Protein (mg/ml)	Specific activity (µmoles/mg/minute)
Control	65.0	2.4	1810
Treated 24 hours	6.1	2.5	160
Treated 18 hours	6.0	2.7	150

Reversal of Inhibition

Table 7 lists the compounds which were used in an attempt to reverse the inhibitory effects of benomyl. These compounds are those for which benomyl might act as an analogue or are a part of a biosynthetic pathway upon which benomyl might exert an inhibitory effect. Serine and cysteine were also included after Mailman et al. (1971) found reversal of benomyl inhibition in S. cerevisiae by thiols. None of the compounds tested had any effect in relieving inhibition of growth.

Effects on Synthesis of Proteins and Nucleic Acids

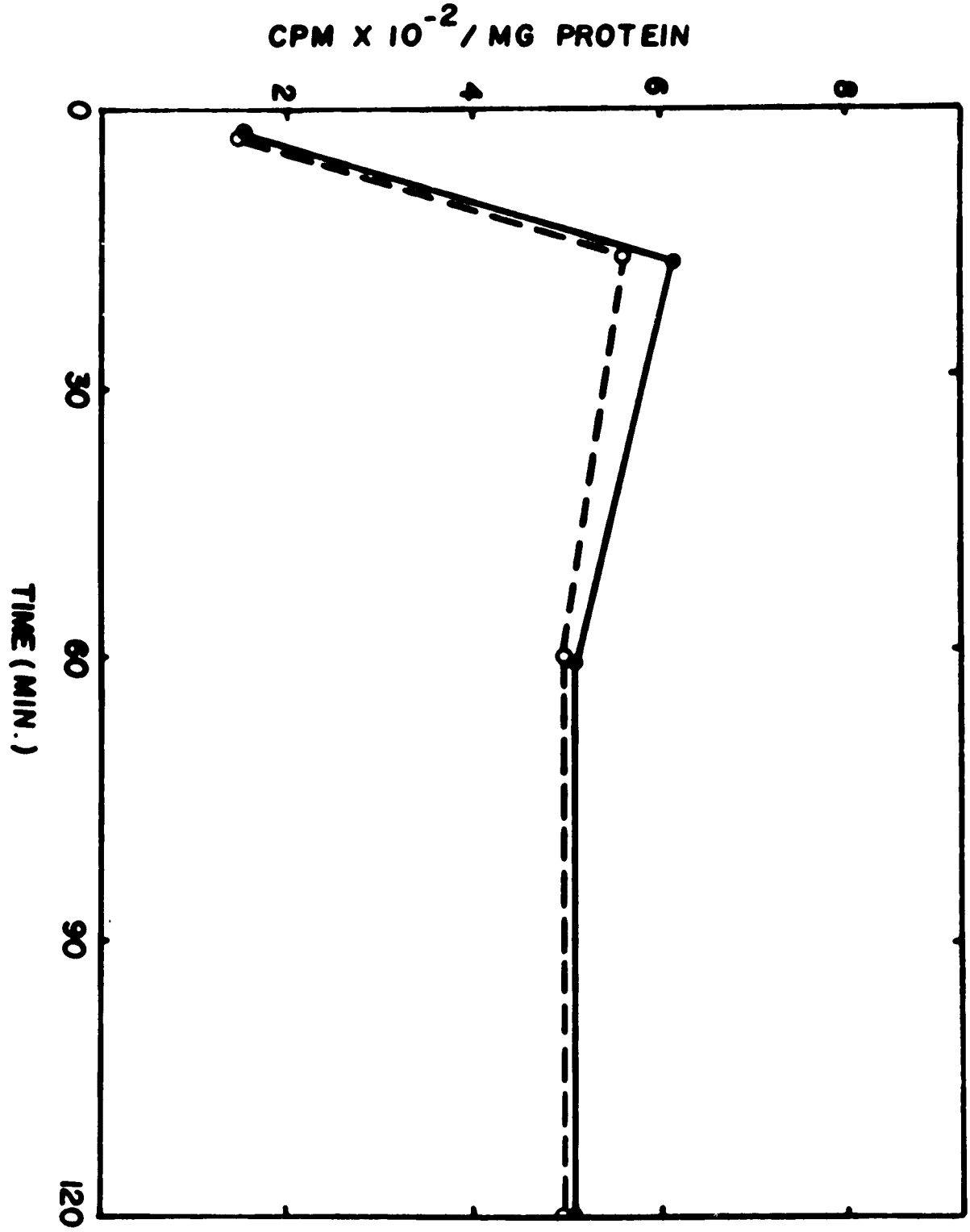
Analysis of proteins in mycelia and cytoplasmic fractions of Neurospora treated with benomyl for 48 hours (Table 2) implied that protein synthesis might well be the target of the toxicant's inhibitory activity. Therefore, it seemed appropriate to determine the effect of benomyl on in vivo incorporation of radiolabeled amino acids into protein. As Figure 16 illustrates, the introduction of benomyl did not

Table 7. Compounds tested for reversal of inhibition by benomyl in *N. crassa*.

Compound	Concentrations used (µg)	Concentration of benomyl (µg/ml)
Adenine	100*	0.5
Adenosine	100*	0.5
ATP	100*	0.5
Guanine	100*	0.5
Folic acid	2, 5, 10	0.5, 1.0
NAD	5, 10, 20	0.5, 1.0
Tryptophan	50, 100, 200	0.5, 1.0
Histidine	50, 100, 500, 1000, 1500	1.0
Vitamin B_{12}	10, 20, 50, 100, 200	1.0
CTP	50, 100, 500, 1000, 1500	1.0
TTP	50, 100, 500, 1000, 1500	1.0
TMP	50, 100, 1000	1.0
Uridine	50, 100, 500, 1000, 1500	1.0
Serine	10, 50, 100, 200	0.2, 0.5, 1.0
Methionine	10, 50, 100, 200	0.2, 0.5, 1.0
Cysteine	10, 50, 100, 200	0.2, 0.5, 1.0

*Concentrations in µg/ml medium; all other values represent total µg applied to center of agar plate.

Figure 16. The effect of benomyl on incorporation of C^{14}-leucine into protein. Benomyl was added after 6 hours preincubation to give 1.0 µg/ml. Symbols: Control (●), benomyl treated (○).


CPM X 10^-2 / MG PROTEIN
2
4
6
8
0
30
60
90
120
TIME (MIN.)

decrease incorporation of label up to 2 hours. These results suggest, then, that the primary site of inhibition is not protein synthesis and that the decrease in proteins observed earlier was a long term, secondary effect.

Clemons and Sisler (1971) reported that MBC reduced incorporation of labeled precursors into DNA and RNA of N. crassa. Consequently, the incorporation of C^{14}-adenine and H^{3}-uridine was utilized to show the effects of benomyl on nucleic acid synthesis by wild type Neurospora as well as purine- and pyrimidine-requiring mutants. Incorporation of C^{14}-adenine into DNA of ad-1 (Table 8) was slightly inhibited by 2 hours but incorporation into RNA was not affected. Because specific activities are low in these samples, it is difficult to attach great significance to the differences between control and treated samples. However, the amount of radioactivity found in the TCA supernatant of the treated cells was 30% higher at 15 minutes and 60% higher at 2 hours, indicating a possible accumulation of label in the TCA soluble fraction.

Table 8. The effects of benomyl on incorporation of C^{14}-adenine into DNA and RNA of ad-1.

Nucleic acid	Sample time	Specific activity (cpm/µg nucleic acid)	
		Control	Treated
DNA	15 minutes	3.0 ± 0.5	2.3 ± 0.3
	2 hours	17.0 ± 1.2	13.0 ± 1.3
RNA	15 minutes	2.4 ± 0.1	2.1 ± 0.1
	2 hours	14.7 ± 0.3	16.2 ± 0.3

When H^3-uridine was used as a precursor (Figure 17), benomyl caused a stimulation of incorporation into both DNA and RNA during the first 30 minutes, but after this time control and treated samples were found to have nearly the same specific activities. This pattern occurred with wild type and the pyrimidine-requiring strain, pyr-3.

Based upon the preceding labeling experiments, it seems unlikely that benomyl produces an immediate inhibition in synthesis of macromolecules. However, it is entirely conceivable that protein and/or nucleic acid synthesis may be affected after a 2-hour period. Indeed, the work of Clemons and Sisler (1971) suggests this is the case. Cultures of control and treated Neurospora were removed at longer intervals after addition of benomyl and processed for extraction and estimation of protein, DNA and RNA by colorimetric methods. Figure 18, in which the results are plotted as per cent of control, establishes that benomyl produced an initial stimulation in formation of DNA followed by a reduction in synthesis to approximately 50% of control at 8 hours. Synthesis of RNA showed much the same results, decreasing to 75% of control at 8 hours. Protein synthesis also was stimulated at first, but dropped after 15 minutes, and final concentration of protein in treated cells was as high as 90-95% of control.

Figure 17. The effect of benomyl on incorporation of H^3-uridine into DNA and RNA. Benomyl was added after 6 hours preincubation to give 1.0 µg/ml. Symbols: DNA control (●), DNA benomyl treated (○), RNA control (■), RNA benomyl treated (□).

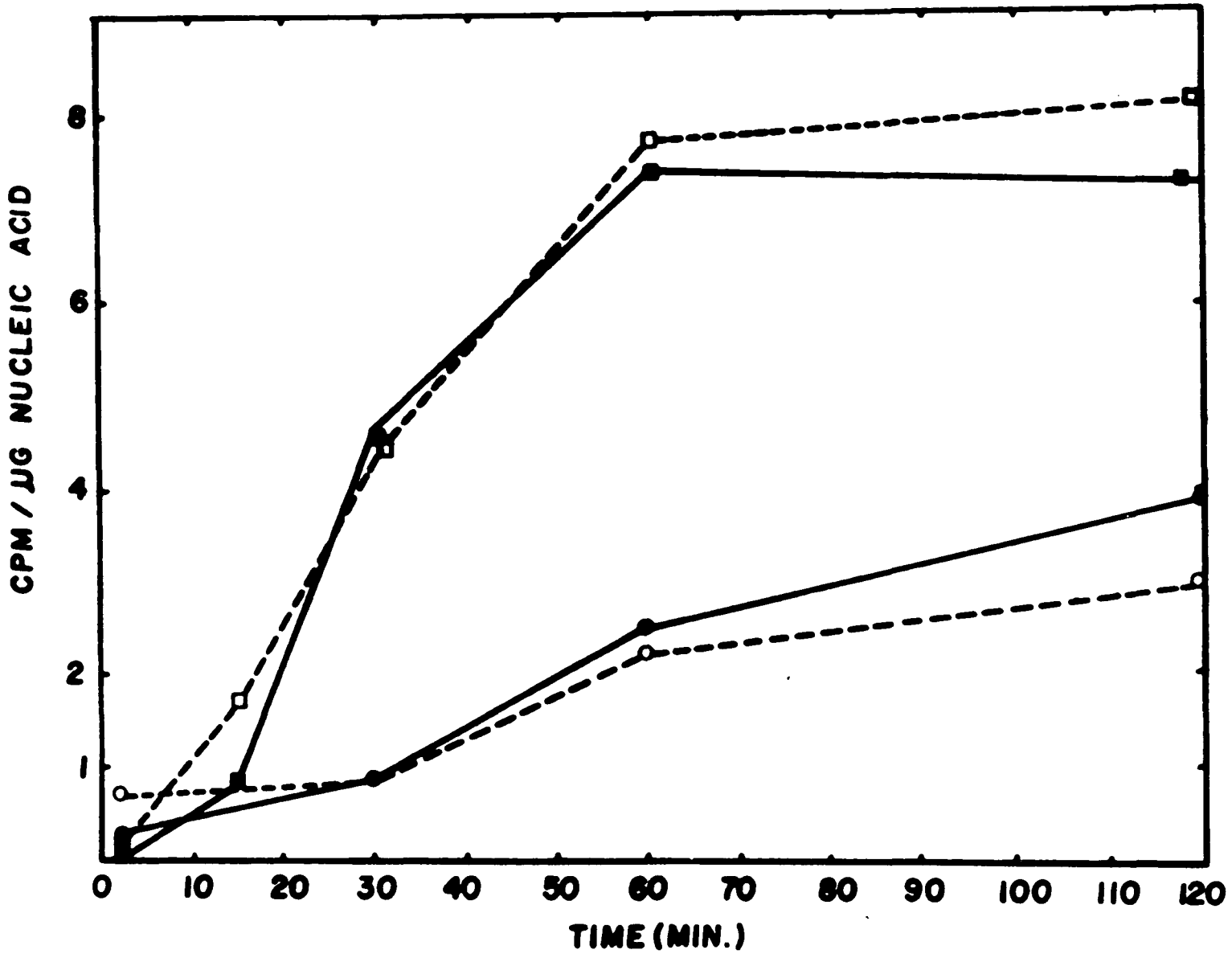
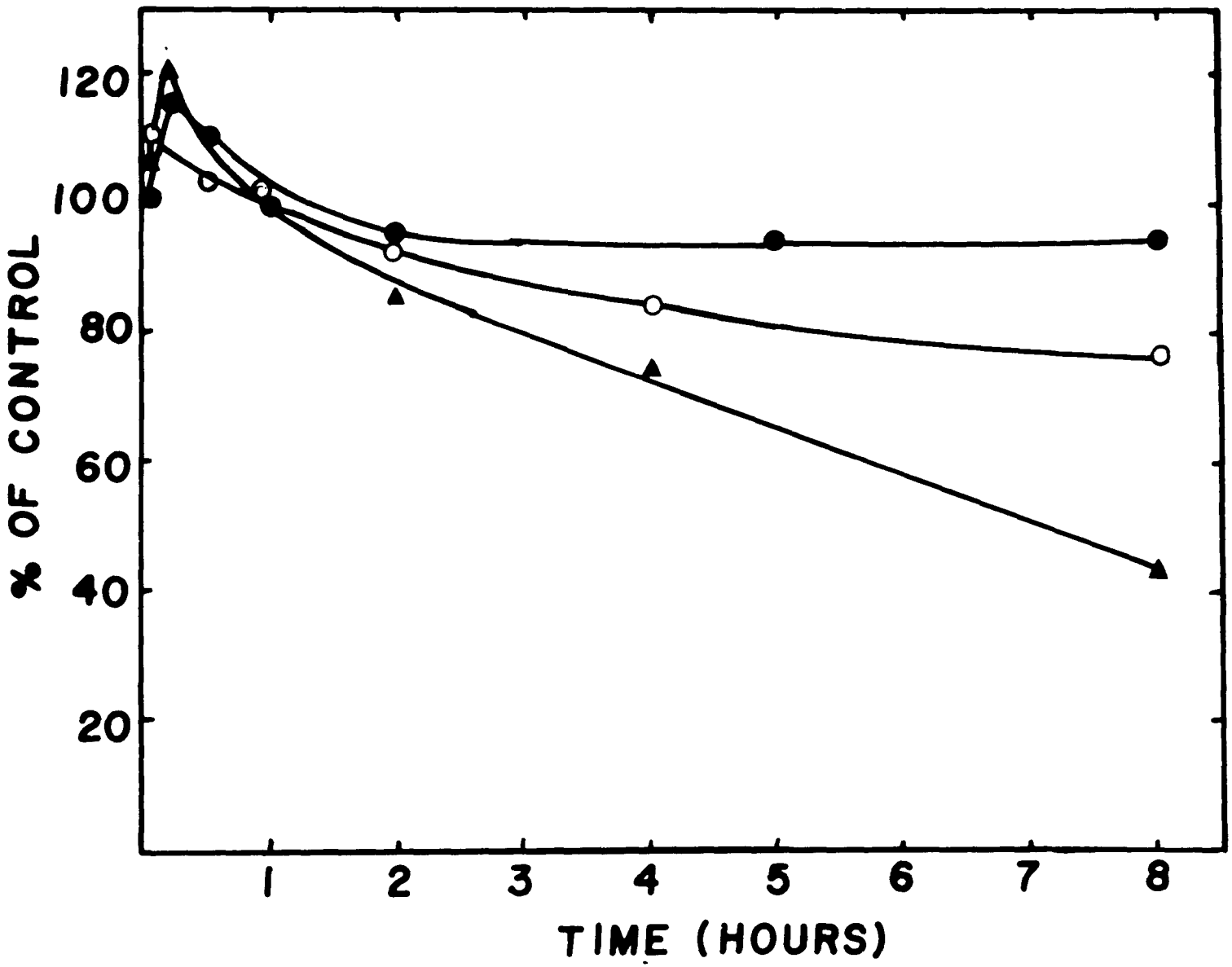

CPM / µG NUCLEIC ACID
8
6
4
2
1
0
10
20
30
40
50
60
70
80
90
100
110
120
TIME (MIN.)

Figure 18. Effect of benomyl on protein, DNA and RNA synthesis in N. crassa. Benomyl (1.0 ug/ml) was added after 6 hours preincubation and samples removed at 15 minutes, 30 minutes, 1, 2, 4 and 8 hours. Protein, DNA and RNA content of benomyl treated cells are plotted as per cent of control. Symbols: Protein (●), RNA (○) and DNA (▲).


120
100
80
60
40
20
% OF CONTROL
1
2
3
4
5
6
7
8
TIME (HOURS)

Double-Labeling Experiments

Since there is an unmistakable lag which preceeds the inhibitory effects of benomyl, it is possible that the actual site of action of the toxicant involves a process prior to the incorporation of nucleotides into nucleic acids. We chose, therefore, to follow the pattern of nucleotide and nucleic acid synthesis over a period of 8 hours after the addition of benomyl to preincubated cultures. Both C^{14}-adenine and H^3-uridine were used as precursors. Table 9 shows the calculated radioactivity present in the homogenates and in the aqueous fraction following phenol extraction. The amounts of C^{14} and H^3 in homogenates of control and treated cells were essentially equivalent. However, at 30 minutes, the levels of radioactivity in the aqueous fraction of the treated sample were nearly 3 times those of the control. At 2 hours, the aqueous portions were comparatively the same, and by 4 and 8 hours, label in water soluble components of the treated samples was considerably reduced. By 4 hours, the ratios of C^{14} and H^3 in the aqueous fraction relative to the total in the homogenate was less in both samples; nevertheless, the ratios in the treated were about one-half those of the control.

Column chromatography on DEAE-cellulose was performed to determine how the label was distributed among bases, nucleosides, nucleotides and nucleic acids. Only the results from the 2 and 8 hour samples are shown. Most of the label at 2 hours appeared in a major peak eluting at 0.7 M NaCl,

Table 9. Distribution of C^{14} and H^3 in various fractions following phenol extraction of homogenates.

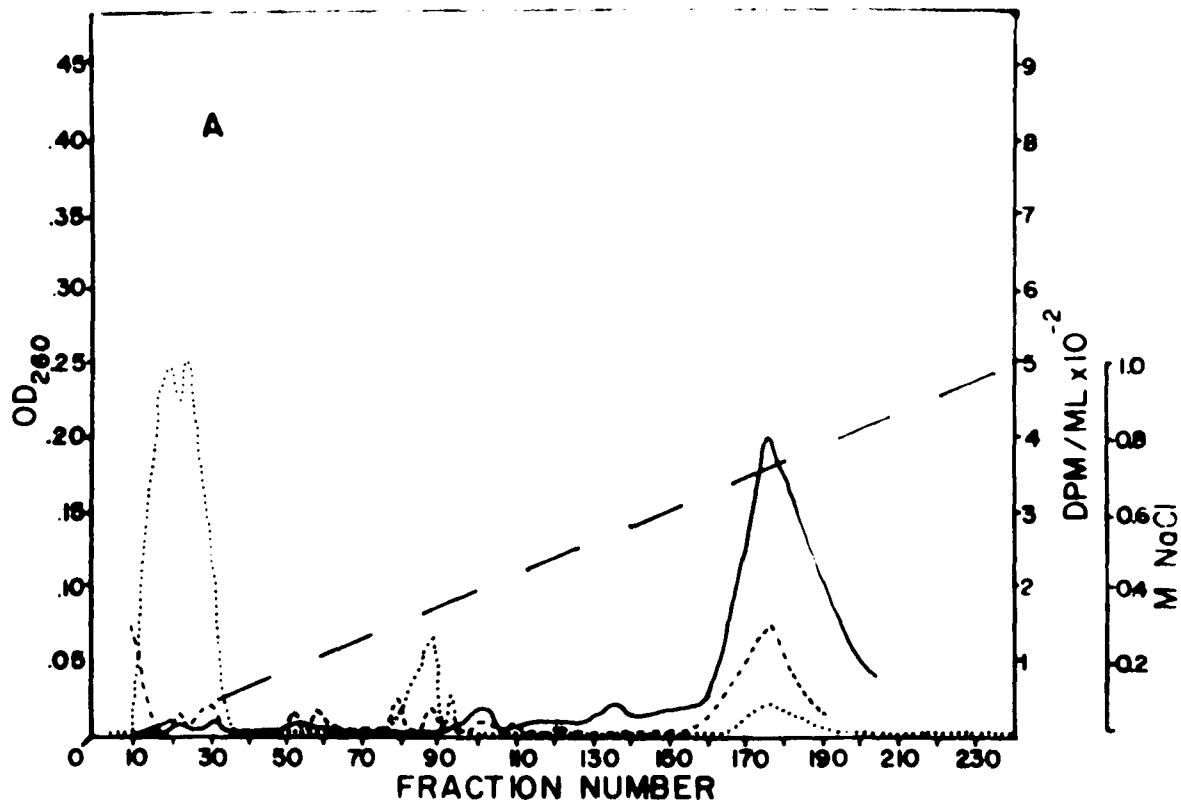
Sample	Total dpm in homogenate/mg dry weight		Total dpm in aqueous fraction/mg dry weight		dpm in aqueous / dpm in homogenate	
	C^{14}	H^3	C^{14}	H^3	C^{14}	H^3
Control 30 min.	36,350	8,730	950	280	0.026	0.032
Treated 30 min.	32,250	9,950	2510	810	0.078	0.081
Control 2 hours	28,630	17,830	740	330	0.026	0.019
Treated 2 hours	27,430	18,980	670	250	0.024	0.013
Control 4 hours	20,050	12,500	130	60	0.006	0.005
Treated 4 hours	21,010	11,890	60	20	0.003	0.002
Control 8 hours	-	-	350	120	-	-
Treated 8 hours	-	-	10	10	-	-

and treated material contained slightly more C^{14} than did control material (Figure 19). Preceding this major peak were several smaller ones, also slightly higher in the treated sample. The unlabeled peaks eluting at 0.05 M and 0.2 M NaCl were substantially greater in the control. In the 8-hour cultures, as seen in Figure 20, the peak eluting at 0.7 M NaCl had virtually disappeared from the treated sample whereas the smaller peaks preceding it had increased in radioactivity relative to the control.

Preliminary identification of the major peaks was determined by comparison to the elution pattern of a group of standards. A mixed sample of known bases, nucleosides, nucleotides and nucleic acids was passed through a DEAE-cellulose column just as described above for experimental samples. Bases and nucleosides eluted between 0.05 and 0.2 M NaCl, nucleotides at 0.3 to 0.4 M NaCl and RNA at 0.6 to 0.7 M NaCl.

At 8 hours the peak eluting at 0.7 M NaCl (Figure 20) was most affected by benomyl, therefore, further experiments were carried out (using fractions 150-190) to ascertain its identity. Measurements of radioactivity, A_{260} and A_{280} before and after dialysis showed that both the control and treated fractions were dialyzable. The A_{280}/A_{260} values of the dialyzed material were 0.59 and 0.79 for the control and treated, respectively, indicating the former was primarily nucleic acid but that the latter contained some protein. Colorimetric analysis of RNA, DNA and protein verified that

Figure 19. DEAE-cellulose column chromatography of the aqueous fractions from homogenates of N. crassa grown for 2 hours in the presence of C^{14}-adenine and H^3-uridine. A, control; B, benomyl treated at 1.0 ug/ml. Symbols: C^{14} (——), H^3 (---), A_{260} (•••), NaCl gradient (— —).


A
OD_{260}
DPM/ML x10^{-2}
M NaCl
FRACTION NUMBER

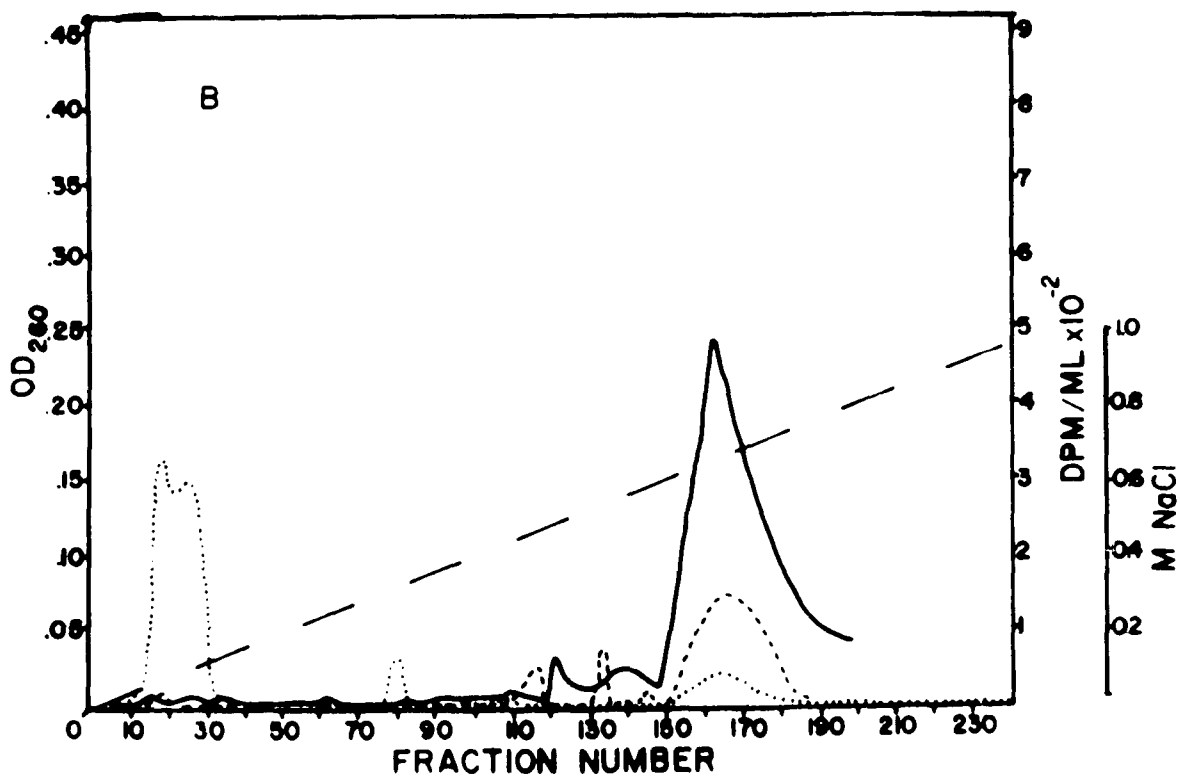
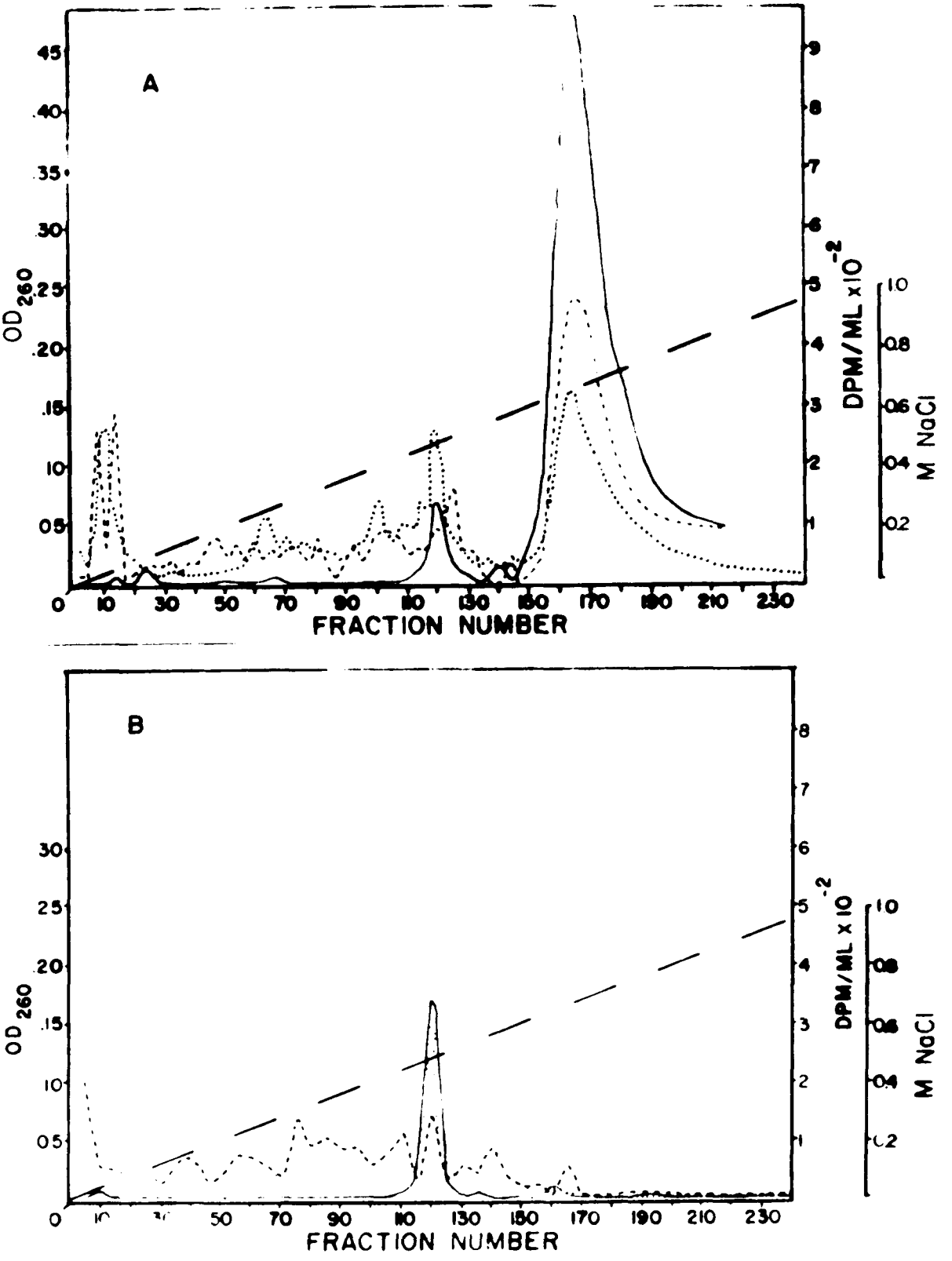

B
OD_{260}
DPM/ML x10^{-2}
M NaCl
FRACTION NUMBER

Figure 20. DEAE-cellulose column chromatography of the aqueous fractions from homogenates of N. crassa grown for 8 hours in the presence of C^{14}-adenine and H^{3}-uridine. A, control; B, benomyl treated at 1.0 µg/ml. Symbols are the same as those described in Figure 18.

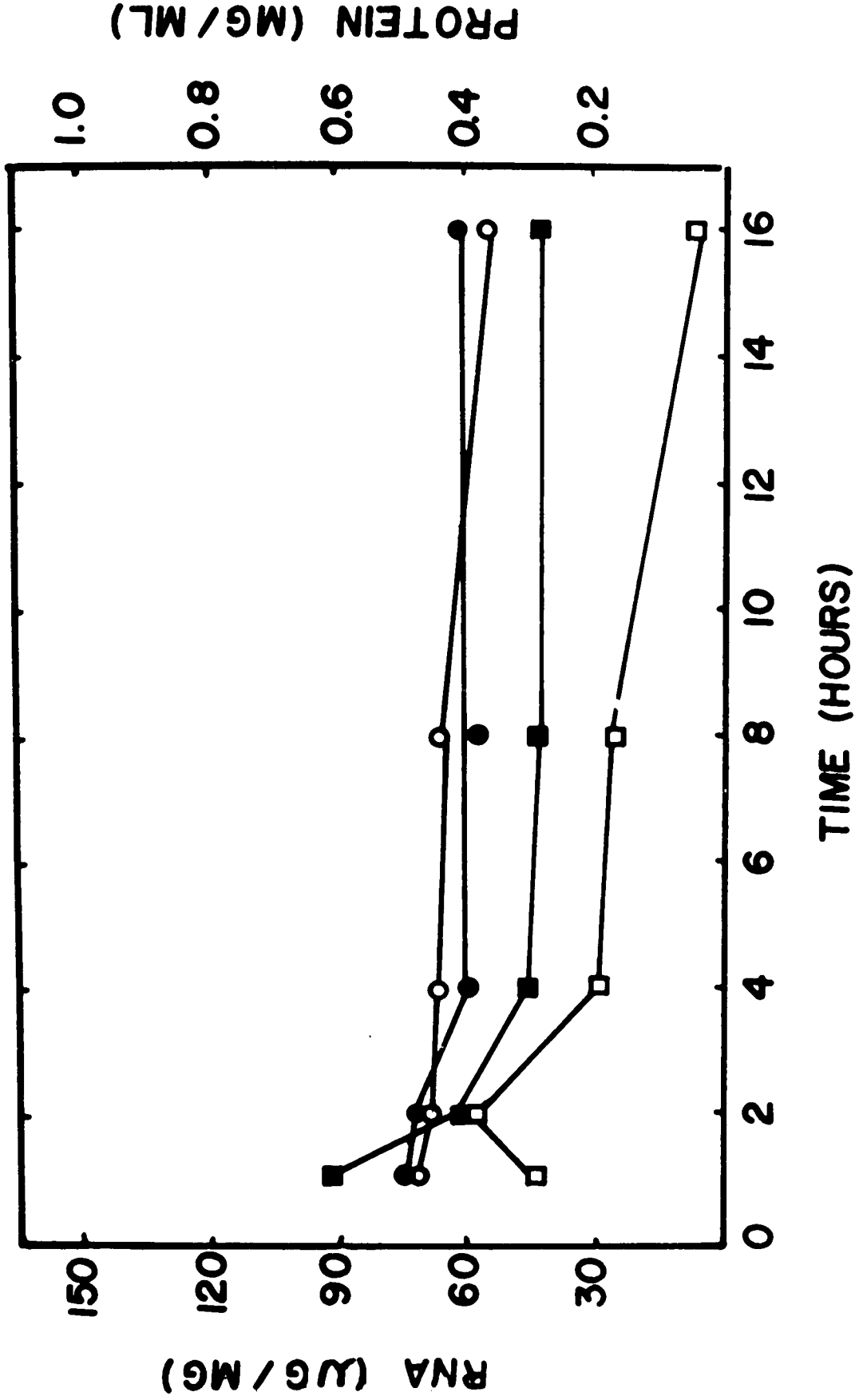

A
B
OD260
DPM/ML x10-2
M NaCl
FRACTION NUMBER

the control fraction was all RNA (706 µg total) whereas the treated was a mixture of RNA (66 µg total) and protein (1.1 mg). No DNA was detected in either fraction.

Biochemical Analysis to 16 Hours

The gradual decrease in RNA and DNA content and in the incorporation of labeled precursors into these nucleic acids in cells treated for 8 hours with benomyl provided evidence that the effects of benomyl in Neurospora were somehow manifested in the metabolism of nucleic acids, but that the inhibition was delayed rather than immediate. Therefore, a series of biochemical tests were performed on cytoplasmic fractions from cultures grown in the presence of the toxicant for periods of 1 to 16 hours. This was done in an attempt to correlate the progressive effects of benomyl upon several aspects of metabolism. The results of protein and RNA assays are presented in Figure 21. Protein content remained unaffected until 14 hours after the addition of benomyl and even at 16 hours inhibition was very slight. RNA synthesis, on the other hand, was suppressed at 30 minutes, recovered at 2 hours, and was then progressively inhibited to 81% at 16 hours. The concentration of DNA in these samples was too low to be detected colorimetrically. With the exception of the 1-hour sample, these observed effects on protein and RNA synthesis were similar to the results obtained using a different procedure (Figure 18).

Figure 21. The effect of benomyl on protein (mg/mg dry weight) and RNA (µg/mg dry weight) synthesis in N. crassa 74A. Cultures were grown in the presence of 1.0 µg/ml benomyl for 1 to 16 hours. Benomyl was added after 6 hours preincubation. Symbols: Control protein (●), benomyl treated protein (○), control RNA (■), benomyl treated RNA (□).


PROTEIN (MG/ML)
1.0
0.8
0.6
0.4
0.2
RNA (UG/MG)
150
120
90
60
30
0
2
4
6
8
10
12
14
16
TIME (HOURS)

The levels of total phosphorus at 1 hour (Figure 22) were 5.8 and 7.0 μmoles/mg lyophilized dry weight in control and treated samples, respectively. By 2 hours, both samples contained approximately 5 μmoles/mg, a level which was maintained up to 16 hours. Measurements of labile and extra-labile phosphorus produced identical results, and only data from the determinations of labile phosphorus are shown (Figure 22). At 1 hour, the values were higher in the treated sample; at 2 hours they were equivalent to those of the control, but increased again between 8 and 16 hours. At 16 hours labile phosphorus in treated samples coincided with decreases in RNA; it is possible the two phenomena are associated. Estimations of total hexose (Figure 23) revealed that benomyl caused a reduction in total hexose content of the cytoplasm by 1 hour. In treated cultures, hexose content increased with time, but at a slower rate so that at 16 hours the concentration of total hexose was 67% of control.

Extraction of Bases, Nucleosides and Nucleotides

From the previous results it seemed likely that nucleic acid synthesis was indeed affected by benomyl. However, the quantitation of bases, nucleosides and nucleotides was inconclusive. Therefore, under the assumption that the synthesis of nucleotides was in some way altered in the presence of benomyl, another experiment was designed to measure the levels of nucleic acid precursors. The elution patterns of control and treated (48 hours) samples from DEAE-cellulose

Figure 22. The effect of benomyl on total phosphorus and labile phosphorus content (μmoles/mg dry weight) of N. crassa 74A. Cultures were grown as described in Figure 21. Symbols: Total phosphorus of control (●) and benomyl treated (○); labile phosphorus of control (■) and treated (□).

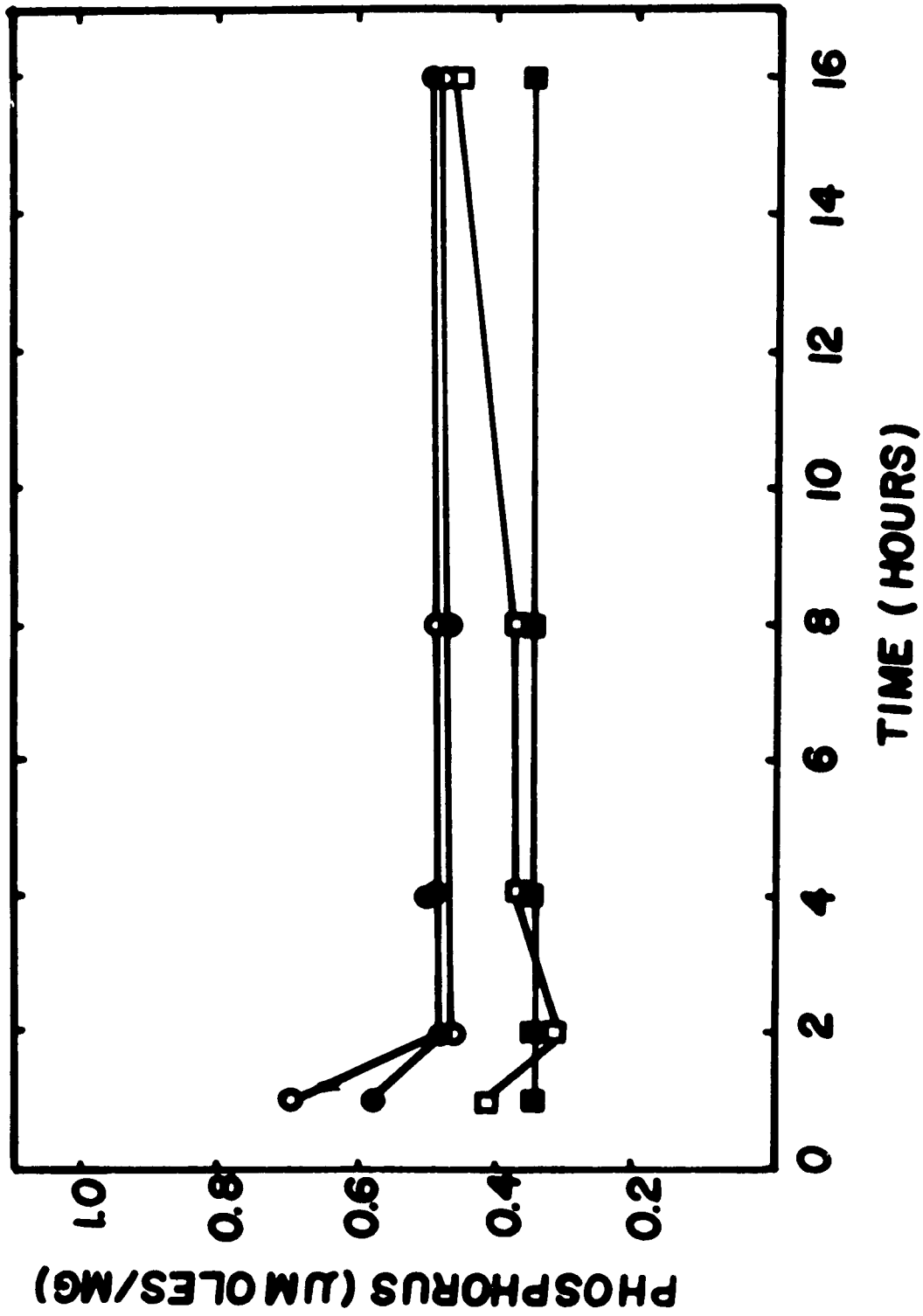
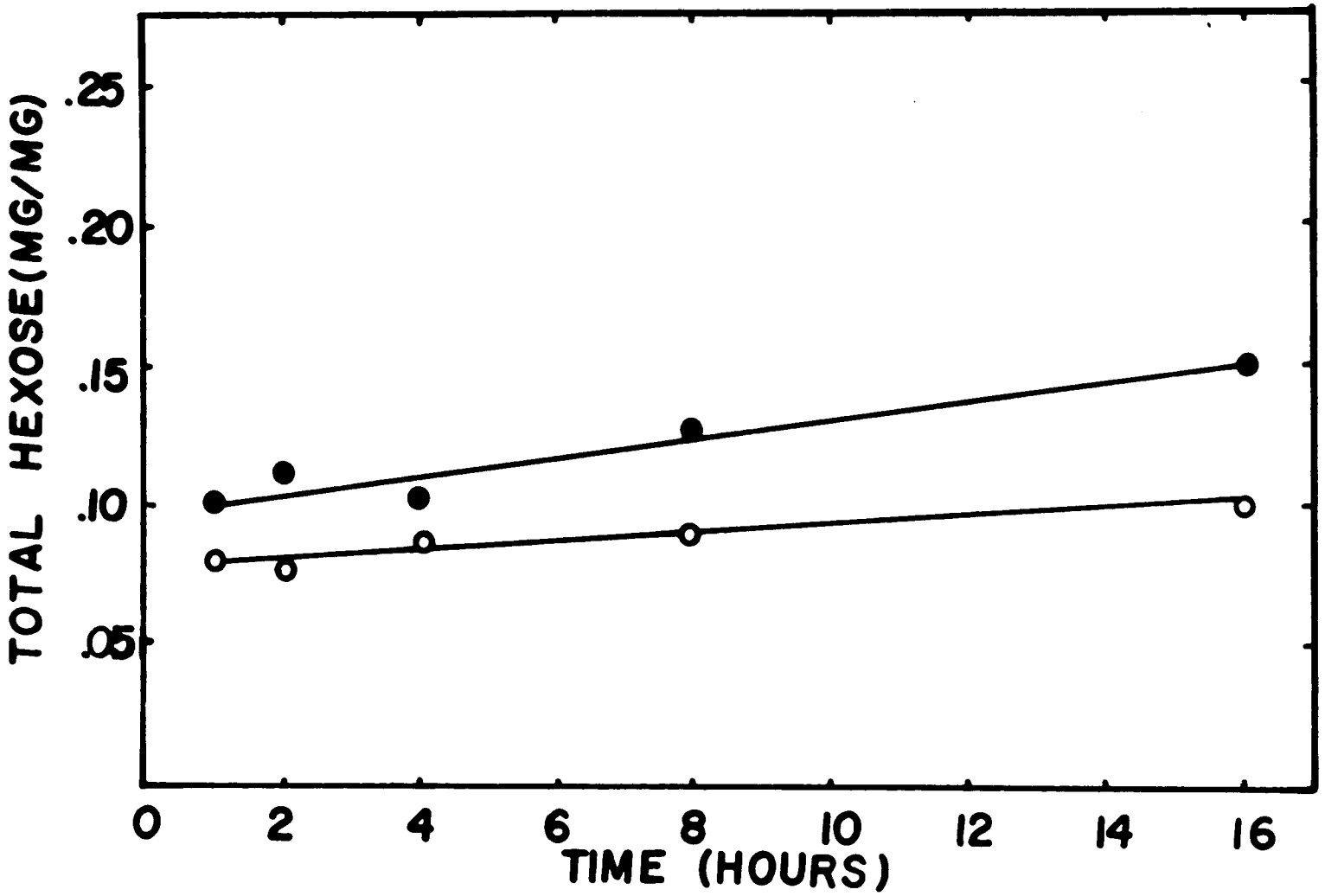

PHOSPHORUS (UM OLES/MG)
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
10
12
14
16
TIME (HOURS)

Figure 23. The effect of benomyl on total hexose content (mg/mg dry weight) of N. crassa 74A. Cultures were grown as described in Figure 21. Symbols: Control (●), benomyl treated (○).


TOTAL HEXOSE(MG/MG)
.25
.20
.15
.10
.05
0
2
4
6
8
10
12
14
16
TIME (HOURS)

columns (Figure 24) demonstrate that the concentrations of bases and nucleosides (peak II) were much lower in benomyl treated cells. The levels of nucleotides in peak V were equivalent but those in peak IV were lower in the treated sample. Only very small amounts of nucleic acid were eluted with 1 M NaCl, probably due to the strong binding forces between the DEAE-cellulose and nucleic acid. Chromatography of peak V in isobutyric acid:NH_4OH:H_2O revealed four components from the control sample and five from the treated. Quantitation of each of these components and their probable identities, which were determined by spectra, chromatography in another solvent and electrophoresis, are given in Table 10. Components C_2 and T_2 have been tentatively classified as UDP. UDP-glucose (UDPG) was found exclusively in treated samples as was a component which fluoresced under ultraviolet light. This fluorescent material could be of particular interest since it chromatographed with adenosine in both solvent systems. Results shown indicate that benomyl induced changes in the distribution of adenine and uridine nucleotides such that quantities of AMP, ADP and UMP were reduced and, possibly, UDP (C_2 and T_2) was increased.

Figure 24. DEAE-cellulose column chromatography of N. crassa nucleic acids and nucleic acid precursors. Benomyl (1.0 μg/ml) was added at 6 hours and cultures were allowed to grow in the presence of the toxicant for 48 hours. The aqueous portion resulting from a phenol extraction of mycelia was passed through the column. Control (---), benomyl treated (——).

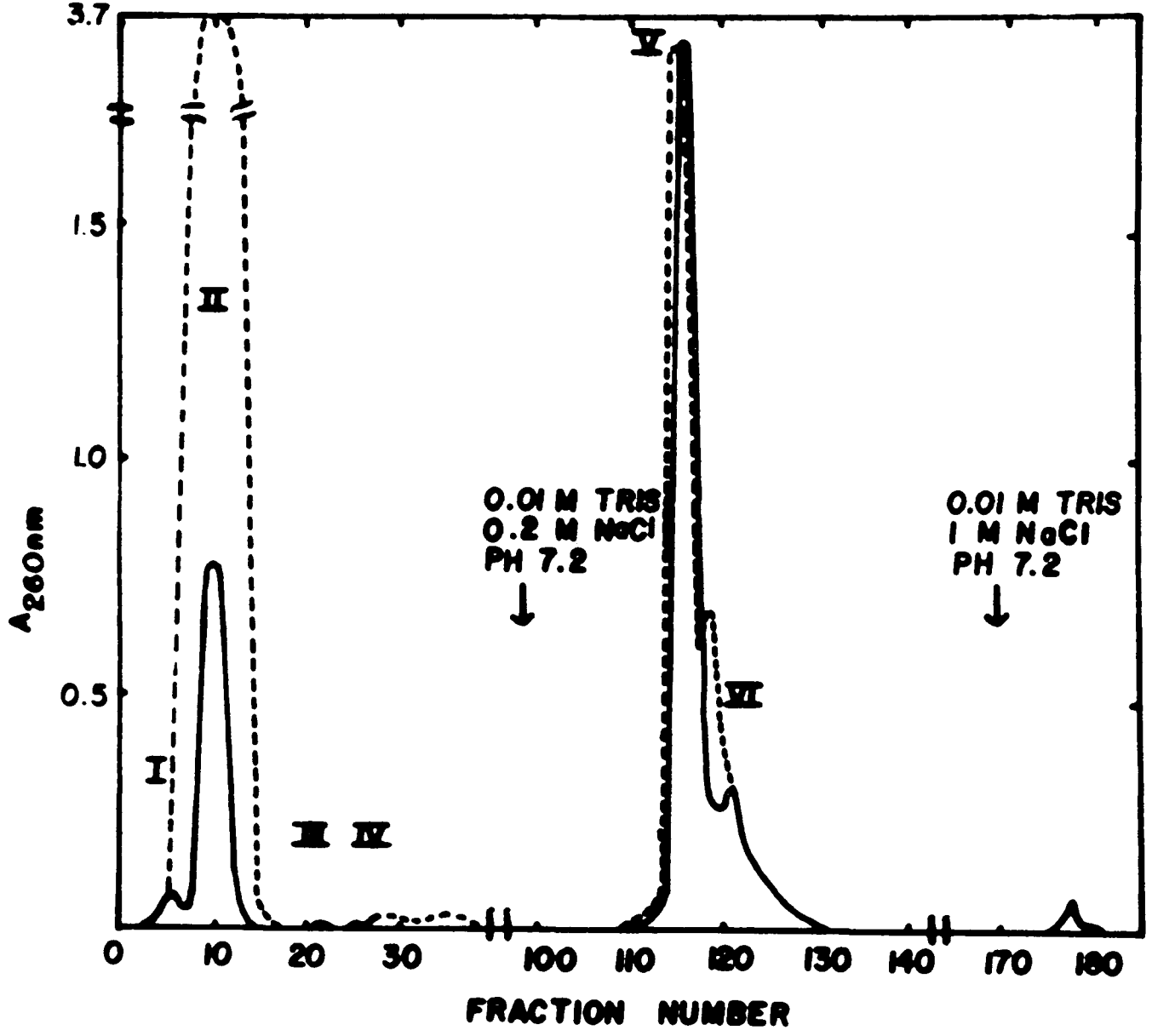

3.7
1.5
1.0
0.5
A_{260nm}
I
II
III IV
V
VI
0.01 M TRIS
0.2 M NaCl
PH 7.2
0.01 M TRIS
1 M NaCl
PH 7.2
0
10
20
30
100
110
120
130
140
170
180
FRACTION NUMBER

Table 10. The effect of benomyl on distribution of nucleotides in N. crassa. C, control; T, treated; Fl, fluorescent component.

Components of peak V found by chromatography	$\frac{A_{260} \text{ of eluted material}}{A_{260} \text{ of material applied}}$	Probable identities of components
C_1	0.16	UXP
T_1	0.11	UXP, UDPG, Fl
C_2	0.37	UXP
T_2	0.67	UXP, Fl
C_3	0.19	UMP
T_3	0.08	UMP, UDPG, Fl
T_4	0.02	-
C_4	0.28	AMP, ADP
T_5	0.11	AMP, ADP, Fl

Using chromatography and ultraviolet spectra, the components of peak II from control and treated samples (Figure 24) were similarly determined (Table 11). Remarkably, uracil was absent from the treated samples and uridine appeared to be dramatically reduced. Adenosine (or adenine), which was present in relatively large quantity in the treated sample was not in the control. Again, a fluorescent component was found from the benomyl treated cells which had the same mobility in isobutyric acid:NH_4OH:H_2O as adenosine. The concentration of T_1 was too low for ultraviolet spectrum and further chromatography, but it traveled near uridine and guanosine in isobutyric acid:NH_4OH:H_2O. Classification of

this component will be withheld due to inconclusive data.

Table 11. The effect of benomyl on distribution of bases and nucleosides in N. crassa. C, control; T, treated; Fl, fluorescent component.

Components of peak II found by chromatography	$\frac{A_{260} \text{ of eluted material}}{A_{260} \text{ of material applied}}$	Probable identities of components
C_1	0.70	Uridine
T_1	0.05	-
C_2	0.31	Uracil
T_2	0.94	Adenosine or adenine
T_3	-	Fl

The Effect of Benomyl on Ascospore Segregation

Several research groups have postulated that MBC and benomyl inhibit cell division in yeasts (Hammerschlag and Sisler, 1973) and fungi (Clemons and Sisler, 1971; Davidse, 1973). Therefore, experiments were designed to determine whether division was affected in Neurospora. Black ascospores produced in a cross contain a normal genome and are viable spores. White spores are aberrant and non-viable, thus an increase in the number of white ascospores from a cross signifies a malfunction in the process of ascospore formation. The data presented in Table 12 suggest that benomyl causes an increase in white spores; however, these results must be interpreted with caution since the results are often erratic. Apparently, the methods for determining per

cent viability are not reliable and those values should be disregarded.

Table 12. The effect of benomyl on ascospore segregation in N. crassa.

Concentrations (µg/ml) of benomyl on which crosses were carried out	# Black ascospores / # White ascospores	% Viability
0.0	22.8, 25.7	79, 57
0.1	27.3, 34.0	58, 86
0.125	19.6	91
0.15	24.7, 16.7	32, 65
0.175	16.6	74
0.20	17.7, 13.9	48, 86

Mutagenic Effects of Benomyl Treatment

The method of filtration enrichment was employed for the selection of auxotrophic mutants following treatment with concentrations of benomyl which would just permit growth. The calculated mutation rates at 62-hour sampling time (Table 13) imply that benomyl may be slightly mutagenic. However, at 70, 82 and 88 hours, the number of mutations in treated samples were no greater than in the control.

Table 13. Mutagenic effects of benomyl as determined by filtration enrichment.

Sampling time (hours)	Mutation rate (x 10^8)		
	Control	Benomyl treated 0.27 μg/ml	0.29 μg/ml
62	7.6	17.6	12.8
70	6.4	13.6	4.0
82	1.6	2.8	2.4
88	4.8	1.2	2.0

Genetics of Resistance

Although 40 benomyl-resistant mutants were isolated, 15 of these were chosen for complete mapping procedures such that these 15 would be representative of both mating types as well as *hist*$^+$ or *hist*$^-$ phenotypes. The characteristics of these mutants are listed in Table 14.

Eight of the mutants were crossed to suitable mating types of *alcoy* strain for preliminary linkage tests. *Alcoy* carries three reciprocal translocations with readily "visible" markers near the interchange points so the presence of the gene in question on either of the linkage groups involved in the translocation can be discerned by linkage to the marker. The marker for the I:II translocation is *al* (albino), the one for IV:V is *cot-1* (colonial temperature sensitive), and the one for III:VI is *ylo-1* (yellow). The results of the crosses are listed in the Appendix, Table 1, and clearly show that benomyl resistance is linked to *ylo-1*,

Table 14. Characteristics of benomyl-resistant mutants used in mapping studies.

Number of mutant	Mating type	Histidine dependence
511	a	-
519	A	-
526	a	-
5210	a	+
557	A	-
572	A	+
586	A	+
5810	A	-
5102	A	+
5107	a	-
5128	A	+
111	A	-
123	a	+
128	A	-
159	A	-

+ requires histidine supplement

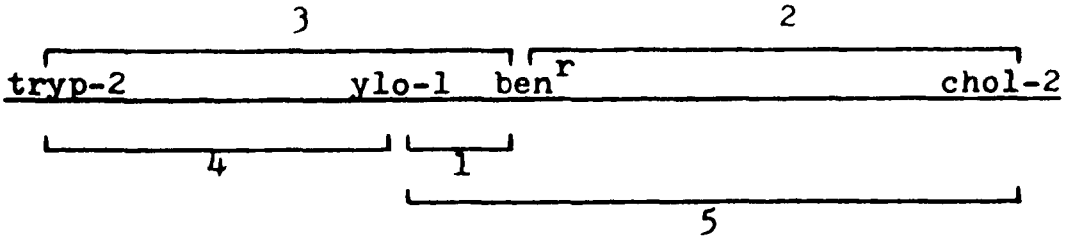
- does not require histidine in the medium

i.e., all orange ascospore isolates are resistant and all yellow isolates are sensitive. Resistance relative to cot-1 or to al shows 50% segregation and therefore no linkage. From this cross, then, the benomyl-resistant locus can be assigned to either linkage group III or VI. Because all 8 mutants gave the same result, it was deemed unnecessary to cross the remaining 7 with alcoy.

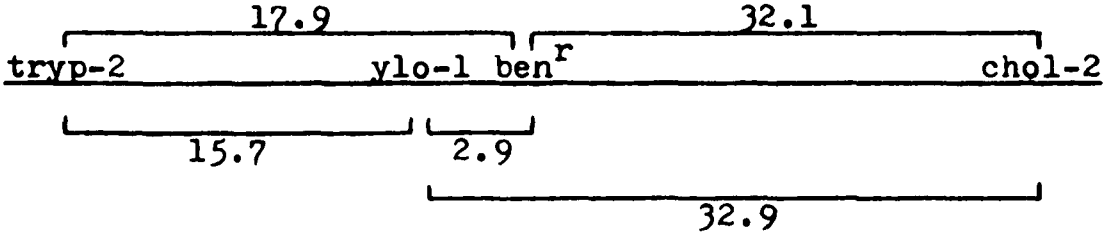
The assignment of the resistant gene to a specific linkage group was accomplished via a cross with strains 1207 and 1208 which carry a marker tryp$^-$ on linkage group III and ylo-1 on linkage group VI. Phenotypes of the ascospores from these crosses are presented in the Appendix, Table 2. Here again the results definitely show linkage between benomyl resistance and ylo-1 on linkage group VI.

Actual map location of the resistant gene was determined from crosses of the mutants to Neurospora strains 2091 and 2092, which possess three markers on linkage group VI (tryp-2, ylo-1 and chol-2). It is known that the sequence of these markers on the chromosome is tryp-2... ylo-1...chol-2. For convenience in determining recombinants, the benomyl-resistant allele was arbitrarily placed so that the sequence would be tryp-2...ben^r...ylo-1... chol-1. Then, recombinants were scored in sets of three, i.e., between tryp-2, ben^r and ylo-1, between tryp-1, ben^r and chol-2 and between tryp-2, ylo-1 and chol-2. The data from all these crosses are shown in the Appendix, Tables 3,

4 and 5. From the results obtained, it became obvious that ben^r was not situated between tryp-2 and ylo-1, but instead was between ylo-1 and chol-2 and was very near ylo-1. The map was rearranged, therefore, and regions were numbered for convenience as follows:



A summary of the map distances in each region determined from all crosses is listed in Table 15. Taking the averages from all, the map units can be assigned as such:



Thus benomyl resistance is quite closely linked to ylo-1 in linkage group VI of Neurospora.

Table 15. Map distances between the *ben*^{r} allele and marker alleles on linkage group VI in *N. crassa*.

Mutant	Region				
	1	2	3	4	5
159	1.8	32	19	17	34
128	0.0	32	10	10	32
5810	3.5	29	15	12	32
5107	3.0	26	18	14	27
519	2.9	34	15	12	33
526	3.1	32	20	17	35
111	2.5	30	15	14	31
5102	2.4	34	23	21	35
5210	4.8	31	16	11	31
572	3.2	32	18	15	35
123	3.8	39	18	15	36
5128	3.0	32	18	19	31
586	1.8	33	24	25	35
557	3.3	26	22	19	27
511	4.8	39	18	15	39

Once map distances were obtained, the mutants were tested for tolerance to increasing concentrations of benomyl. This was done to ascertain whether resistance, even though it was apparently due to a single locus, was manifested to the same degree in all mutants. Table 16 illustrates that the

resistant strains do not withstand the same concentrations of benomyl in the medium. Of the 15, mutants 128 and 519 are the most resistant and 557 is the least resistant.

Table 16. Growth rates (mm/day) of benomyl-resistant mutants on solid medium containing various concentration of benomyl.

Number of mutant	Concentration of benomyl (μg/ml medium)											
	0	0.2	0.5	1.0	1.5	2.0	2.5	3.0	5.0	7.0	10.0	15.0
511	92	100	95					80	70			
516	40			38		38		39	28			
519	82			78				68	40	35	20	
526	100			85			98	55	38	0		
5210	30			25				10		2	0	
557	65	60	55	35	19	0						
572	50		41	40	40	31	22	0				
586	40		35	30	28	23	0					
5810	95	95	95	95				50	45		0	
5102	66			62				15	0			
5107	70		85	80	70	55	35					
5128	85			65				40	30	11	0	
111	55			48		38		10	0			
128	42			32							22	18
159	90			70		50		40	35	0		

DISCUSSION

The inhibitory activity of benomyl in N. crassa seems to require the dynamic processes of growth, or hyphal elongation. Results of growth curves and photomicrographs of cultures indicate that benomyl does not prevent initial germination, but once germ tubes have emerged, they are susceptible to the toxic effects of the fungicide. Germinating conidia are more sensitive to equivalent concentrations of benomyl on solid medium than in liquid shaking cultures. The increased effect on static cultures was also observed by Decallone and Meyer (1972) who studied benomyl inhibition of F. oxysporum.

The time lag which occurs before inhibition of growth becomes evident provokes several possible explanations. First, the toxicant may not enter the cell immediately due to a permeability barrier. Secondly, a concentration effect inside the cell may be necessary to produce an observable change in metabolism; that is, there may be a threshold level of benomyl required for inhibition so the lag represents the time during which benomyl accumulates in the cytoplasm. Although this project did not encompass any studies concerning the permeability properties of benomyl, others have reported that fungicides in general are taken up quite rapidly (Miller, 1959). Absorption of thiabendazole occurs during the first 6 minutes of incubation (Gottlieb and Kumar, 1970) and

MBC is assimilated to a maximum concentration by cells of N. crassa within 30 minutes after addition of the toxicant (Clemons and Sisler, 1971). Decallone and Meyer (1972) have found that benomyl is absorbed by conidia of F. oxysporum so quickly that 70% of maximum uptake takes place in 15 minutes and 90% by 45 minutes. Therefore, it seems unlikely that permeability is a factor in the lag time observed with benomyl, and because maximum concentrations of the toxicant are probably attained within 30 minutes, it is also doubtful that the lag is due to time required for intracellular accumulation of benomyl to threshold levels.

More tenable explanations for the delay in inhibitory effects depend partly upon the age of the cultures and the time at which benomyl is added. If the fungicide is added at the time of inoculation, an increase in absorbance or dry weight does not occur for several hours. This is because conidia are in the process of germination; germ tubes are short and there is little gain in cell mass. After 2 to 3 hours, differences in germ tube elongation become more detectable by the assay methods used. If benomyl is added after germination is complete, then hyphal elongation, which proceeds at a rapid rate, can be more discriminately measured by increase in absorbance, and the time lag is slightly shorter. However, if dry weights are determined, no change is noted until after 4 hours. This phenomenon is apparently due to a continuation in synthesis of cell wall material which provides enough weight (in the initial stages) to

overcome the inhibitory effects on cytoplasmic synthesis.

In spite of the differences in lag times dependent upon how growth curves were done, the delayed effect was present. Further explanations for this delay will be offered in the discussion concerning metabolic changes.

Electron micrographs revealed that the internal organization of benomyl treated Neurospora was considerably modified. The structure of the endoplasmic reticulum became disoriented and dispersed as was seen in cells of B. fabae treated with benomyl (Richmond and Pring, 1971). Treated cells of wild type and slime usually contained a number of large vacuoles, some of which appeared to have cytoplasmic material within. The formation of vacuoles might be a reaction to variations in osmotic pressure inside the cell, resulting from changes in control of permeability at the plasma membrane. Periodic A_{260} and A_{280} measurements of the medium from cultures of wild type and slime treated for 24 hours exhibited no increase in materials absorbing at these wave lengths. There was also no increase of phosphorus in the medium. By these criteria, then, there was no leakage of cellular components into the external medium which would indicate damage to the plasma membrane. Labeling experiments have substantiated this conclusion since there was no inhibition in entry of labeled precursors into the cells grown in benomyl. The possibility of water influx into the treated hyphae can not be disregarded; however, Robertson and Rizvi (1968) maintain that Neurospora does not respond to changes

in osmotic pressure by development of vacuoles but instead it branches subapically. It seems more feasible that these vacuoles represent an increase in autolytic activity due to incipient cell death. One must keep in mind that the ultrastructural changes observed here are the result of 24 hours incubation in benomyl and may only be secondary effects. For a clearer picture of the immediate consequences of benomyl treatment, studies should be carried out on samples taken from cultures at earlier intervals after addition of the toxicant.

Electron micrographs of treated mycelia also illustrate the increase in cell wall thickness surrounding a hyphal structure which has become misshapen and bulbous. Similar morphological distortions due to fungicidal action have been reported by other researchers (Gottlieb and Kumar, 1970; Clemons and Sisler, 1971). Betina et al. (1966) also found distended, irregular hyphae when Aspergillus fumigatis was treated with cyanein, and they postulated these changes were related to alterations in morphogenesis via inhibition of nucleic acid and protein synthesis. Bent and Moore (1966) saw malformed hyphae in Botrytis allii treated with griseofulvin. In N. crassa the thickness of the cell wall enveloping the hyphal strand suggests that new wall material is laid around the entire perimeter rather than at the hyphal tip as in normal elongation (Bartnicki-Garcia, 1968; Hunsley and Burnett, 1970). The bulging of cells may, therefore, reflect an interference with the control of hyphal extension

such that, in effect, the cells are growing in all directions at once.

Spitzenkörper, which are small vesicles usually found at the hyphal apex and are presumed to function in wall synthesis (Grove and Bracker, 1970) were often seen at various locations in treated cells. This also implies that cellular control of elongation is altered by benomyl, although the effect may well be a secondary one. From the micrographs it seems likely that cross wall formation, too, is affected by the presence of the toxicant. Often, lateral cell walls were pinched together with coincident accumulation of cell wall material. Occasionally narrow passageways were observed which would allow cytoplasmic streaming, but normal septal pores were not detected.

Biochemical analysis of mycelia verified that continual synthesis of cell wall occurs in the presence of benomyl with concurrent reduction in cytoplasmic constituents. The increase in total hexose of the mycelia is attributable to the increase of sugars in wall material, since total hexose of the soluble fraction remains lower in treated cells. It is apparently glucose and glucosamine which are incorporated to a great extent into walls of treated hyphae, and this is reflected in the increased amounts of glucosan and β-1,3-glucan polymers. Chitin synthesis is slightly reduced as a result of benomyl treatment. Modifications in cell wall composition undoubtedly have an effect on the function of the wall as well as the morphology of the fungus. Several

investigators have shown that changes in wall composition are responsible for colonial morphology in Neurospora (deTerra and Tatum, 1963), and these changes most frequently occur in fraction I (Mahadevan and Tatum, 1965). This is an interesting point since low concentrations of benomyl can induce growth of Neurospora resembling some colonial mutants, and the most radically altered fraction in benomyl treated cells is fraction I (Table 2). Reissig and Glasgow (1971) have found a galactosamine mucopolysaccharide which has a regulatory function upon growth of N. crassa and causes the production of large single vacuoles; it is assumed to be the same galactosamine polymer as that in the cell wall. The accumulation of such a mucopolysaccharide during abnormal wall synthesis in treated cells might explain the appearance of numerous vacuoles.

The sustained synthesis of cell walls in spite of the eventual inhibition of protein and nucleic acid synthesis is not unique to benomyl-grown Neurospora. Smith and Marchant (1969) found that Rhodotorula glutinis (yeast) grown in the presence of chloramphenicol continued to produce normal wall material which resulted in expanded cell walls with areas of local thickening. A similar phenomenon occurred when protein synthesis in Streptococcus faecalis was halted by chloramphenicol or amino acid starvation (Shockman, 1965). Also, Threlfall (1972) found that the effects of pentachloronitrobenzene in Aspergillus nidulans were manifested through enlarged cell walls and an increase in hexosamine. It is

intriguing to speculate that benomyl might inhibit growth through interference with synthesis and/or assembly of cell wall components. A qualitative analysis of cell walls of selected fungal species has shown that those fungi which contain galactose and, in most cases, galactosamine as wall constituents are also benomyl sensitive (Crook and Johnston, 1962; Bollen and Fuchs, 1970). The resistant species lack galactosamine and contain little or no galactose. The modification in glucosamine and galactosamine content of benomyl treated Neurospora may indeed be a result of interference with wall formation. Nevertheless, it is doubtful that this is the primary site of inhibition since slime, which does not have a detectable cell wall, is also affected by the toxicant.

In spite of the fact that elongation is severely impeded by benomyl, liquid cultures continue to increase in cell mass, even at high concentrations of the fungicide. The implications are that certain of the cellular metabolic functions remain uninhibited. In fact, these studies have shown, as discussed above, that cell wall synthesis does go on and protein synthesis is maintained for some 16 hours after addition of benomyl. Biosynthesis requires energy; therefore it is unlikely that mitochondrial respiration is a target for inhibitory activity. Contrary to experiments with yeast (Say, 1970), there is absolutely no effect on respiration by concentrations of benomyl equal to or greater than those which inhibit growth. Neither does there appear

to be any alteration in oxidative phosphorylation, since uncoupling activity is usually accompanied by an increase in O_2 uptake, and no such development was detected during respiratory studies on isolated mitochondria in the presence of benomyl (Drs. H. J. Colvin and Elizabeth Eubanks, personal communication). Therefore, although cross resistance between benomyl and thiabendazole has been demonstrated in some organisms (Bartels-Schooley and MacNeill, 1971; Hastie and Georgopoulos, 1971), it is unlikely that benomyl inhibits respiration as does thiabendazole.

The effects of benomyl on protein synthesis are very long term ones, and must be considered secondary effects. Similar results were obtained by Decallone and Meyer (1972) and Clemons and Sisler (1971). The only exception is the immediate inhibition of protein synthesis in the yeast, S. pastorianus, by benomyl (Hammerschlag and Sisler, 1972). In N. crassa, amino acid analysis did not indicate benomyl interference with specific amino acid synthetic pathways. Curiously, three amino acids which were more concentrated in treated mycelia are those which act as feedback inhibitors (lysine, threonine and proline), and two are part of the same anabolic pathway (glutamate and arginine). These facts probably have no relevance to the mode of action of benomyl, but may reflect a disintegration of control mechanisms in treated cells.

The absence of invertase activity is evidently another secondary effect of benomyl and is not the cause of growth

suppression. Cultures which were grown on glucose with various concentrations of benomyl were still susceptible to the fungicide; however, at low levels of benomyl there was some compensatory effect of the glucose indicating invertase inactivation due to the toxicant. Active invertase, a glycoprotein, is formed by addition of carbohydrate to the previously synthesized protein. The manifestations of benomyl treatment upon carbohydrate metabolism may prevent this process, thus resulting in an inactive enzyme.

Benzimidazole has been recognized by many scientists as a purine analogue because its toxicity to yeast and bacteria was reversed by adenine and guanine (Woolley, 1944). In plants, the compound is upgraded to a nucleotide and then incorporated into nucleic acids or coenzymes (Kapoor and Waygood, 1965a,b). Several researchers working with benzimidazole fungicides have thought these compounds also act as purine analogues (Sisler, 1969). Therefore the lag period associated with benomyl toxicity may represent the time required for ribosylation and phosphorylation of the compound to a toxic derivative. If benomyl does act as a purine analogue, addition of purines or their metabolites to treated cultures should reverse inhibition. Using numerous compounds associated with purine (and pyrimidine) biosynthesis, we found no relief of toxicity. These results agree with those of Decallone and Meyer (1972) but not with those reported by Bartels-Schooley and MacNeill (1971). However, other evidence has emerged from our experiments which indicates that

benomyl intervenes in the synthesis of nucleotides.

Incorporation of C^{14}-adenine and H^{3}-uridine into DNA and RNA was not dramatically reduced until 2 hours after addition of benomyl, thus the fungicide does not function by inhibiting incorporation of nucleotides into nucleic acids. According to Weijer and Koopmans (1964), DNA synthesis in germinating conidia requires 4 to 6 hours, but after germination and during rapid hyphal growth, DNA will double almost hourly prior to nuclear division. Since incorporation experiments were carried out on mycelial cultures, a rapid drop in incorporation of labeled precursors would be expected if DNA replication were being directly inhibited and no such reduction was observed. The slight decrease in incorporation of C^{14}-adenine at 2 hours in treated cells (Table 8) may be significant. These data must be interpreted cautiously in view of low specific activities; however, the increase of label in the TCA soluble fraction indicates the accumulation of nucleic acid precursors.

The incorporation of H^{3}-uridine into DNA and RNA was stimulated initially, suggesting that benomyl affected the supply of nucleotides from the pool such that exogenously added uridine was more rapidly utilized in treated cultures. This early increase in incorporation was reflected in the double labeling experiments when both C^{14}-adenine and H^{3}-uridine were rapidly taken up in the aqueous fraction of treated mycelia (Table 9). At 2 hours, both control and treated fractions contained the same amount of label, but at

periods after 2 hours, treated cells had very little label in the aqueous portion indicating the synthesis of nucleic acid had been inhibited. The decrease in radioactivity in the aqueous fraction may mean that the label has been randomized into other pathways due to the block in nucleotide synthesis. Analysis of the material in the major peak from DEAE-cellulose identified the material as a species of RNA. Since it eluted from the column at 0.7 M NaCl, this RNA is probably a molecule of small molecular weight. Presumably it is not tRNA, because an inhibition in synthesis of tRNA would not allow the continuation of protein synthesis. This RNA may be fragmented RNA (due to preparation and nuclease activity) or some essential polynucleotide. The remaining high molecular weight nucleic acids were not measured in this experiment since: 1) loss occurred through precipitation at the ether:water interface during ether extraction of phenol, and 2) most nucleic acids can not be eluted from DEAE-cellulose due to the strong binding forces between the exchanger and large polynucleotides.

Quantitation of unlabeled DNA and RNA in control and treated cultures up to 8 and 16 hours confirmed that nucleic acid synthesis was indeed affected after the 1- to 2-hour lag. When nucleic acids were precipitated with cold TCA followed by base hydrolysis of the RNA and extraction of DNA from the remaining pellet, the following results were obtained: again, there was stimulation of DNA and RNA syntheses succeeded first by inhibition of DNA synthesis and

then of RNA synthesis (Figure 18). Therefore it would seem that DNA is the ultimate target. Clemons and Sisler (1971) have suggested MBC acts in this manner. On the other hand, when Neurospora was sonicated and the cell walls removed prior to precipitation and extraction of RNA and DNA, the results were somewhat different. RNA synthesis was inhibited initially (Figure 21), recovered at 2 hours, and then was rapidly inhibited again. Although the initial decrease may be due to experimental error, this is unlikely, since the assay of labile phosphorus also showed an initial increase. Labile phosphorus is represented by such compounds as ATP, ADP, UDP, ribose- , deoxyribose- , aldose- and fructose-1-phosphate and inorganic pyrophosphate; therefore, the plot of labile phosphorus in treated cells (Figure 22) strongly suggests an increase of these compounds in the cytoplasmic fraction at a time when RNA synthesis is suppressed. Although no special precautions were taken to avoid nuclease destruction, the data appear to be valid (i.e., analysis of RNA did not show erratic results). DNA was not determined in these samples, but it seems that this preparative method isolated RNA which shows slightly more inhibitory effects from benomyl than the preceding one.

The distribution of nucleotides, nucleosides and bases affords the strongest evidence that benomyl causes a reduction in nucleic acid synthesis indirectly by affecting the supply of nucleotides to the pool. Concentrations of AMP

and ADP were reduced by one-half; the content of UMP and possibly UTP was decreased; uracil and uridine appeared to be absent. The large quantity of adenosine (or adenine) present in treated material implies a block in phosphorylation of adenosine. The fluorescent component which possessed chromatographic properties similar to those of adenosine substantiates the possibility that benomyl may be ribosylated and phosphorylated since benomyl itself is fluorescent. Several other purine analogues, such as 8-azaguanine, 6-mercaptopurine and 2,6-diaminopurine are known to affect control of phosphoribosyl pyrophosphate and nucleoside pyrophosphorylases in mammalian systems (Hitchings and Elion, 1963). Benomyl itself might be upgraded to a nucleotide and become incorporated into nucleic acid in a similar manner as 8-azaguanine which has been shown to replace 40% of the guanine in RNA and 1% of the guanine in DNA of Bacillus cereus (Hitchings and Elion, 1963). If benomyl were to become incorporated into DNA, however, it might exert a mutagenic effect like that found with 5-bromouracil and 2-aminopurine. Our results (Table 13) gave little indication that benomyl is mutagenic, and critical examination of the work by Seiler (1972, 1973a,b) and Dassenoy and Meyer (1973) does not support this type of function for the compound.

Alternatively, benomyl may interfere with the synthesis of purine nucleotides by competing for anabolic enzymes, such as the inhibition of orotidine-5'-phosphate decarboxylase by 6-azauracil which is converted to 6-azauridine-5'-

phosphate by the fungus (Dekker, 1966).

The effect of benomyl on pyrimidine metabolism is more obscure. Certainly there is an alteration in the distribution of nucleotides, and the increase in UDPG suggests that excess UDP (tentatively identified as component 2, Table 10) is used to form UDPG which then functions as a donor for glucose (and galactosamine) in cell wall synthesis of the β-1,3-glucan (and the galactosamine polymer).

Hammerschlag and Sisler (1973) and Davidse (1973) have provided some evidence that benomyl inhibits mitosis, and Hastie (1970) postulated that benomyl induced abnormal gene segregation to produce instability among diploids of Aspergillus. In addition, it has been demonstrated that certain carbamates prevent mitosis by interference with spindle formation (Audus, 1964; Banerjee and Margulis, 1969; Hepler and Jackson, 1968) in a manner like the binding of colchicine to microtubules (Borisy and Taylor, 1967). Because the production of ascospores in Neurospora involves both meiosis and mitosis, any increase in production of white spores should indicate abnormal segregation of genes during one of these divisions. Our data shows a slight increase in white spores with increasing concentration of benomyl. However, the percent increase is not large, and the results from several experiments were inconsistent. Attempts to examine nuclear activity by cytological stains were unsuccessful. However, electron micrographs did not reveal

abnormalities in nuclear structure or in the number of nuclei per cell. Therefore, it can be concluded that in Neurospora, benomyl does not act on nuclear division. In Neurospora cell division (or elongation and septum formation) and nuclear division are separate events; thus, even though benomyl inhibits elongation it need not do so by exerting its effect on the nucleus.

The fact that low concentrations of benomyl suppress growth of Neurospora suggests that the toxicant acts as a specific enzymatic inhibitor. Clues to its mode of action might evolve from genetic studies of resistant mutants. We were interested in determining whether resistance was controlled by one or more genetic loci and whether the resistant allele was dominant. Genetics maps of a representative sampling from the total number of resistant mutants isolated have clearly illustrated that one locus is responsible for resistance. This is similar to the genetic control of actidione (cycloheximide) resistance in the Myxomycete, Physarum polycephalium (Dee, 1966). However, it is conceivable that other loci exist in N. crassa since there are eight for actidione resistance in S. cerevisiae (Brusick, 1972) in addition to recessive modifier genes which confer greater degrees of resistance (Wilkie and Lee, 1965). Hastie and Georgopoulos (1971) have discovered two unlinked alleles conferring benomyl resistance in A. nidulans, and both are recessive. Preliminary tests of heterokaryons in

N. crassa containing ben^r and ben^s alleles have indicated that ben^r is dominant since the heterokaryons displayed the same tolerance levels as the haploid mutant. Dominance over the wild type allele is not unusual; Hsu (1963) found two unlinked dominant loci in N. crassa for actidione resistance.

The benomyl-resistant mutants of Neurospora which have been mapped do not show the same tolerance levels, and dosage response curves are biphasic which might imply benomyl has more than one site of action; however, these biphas curves may only represent the permeability properties of the mutants with respect to benomyl.

No attempts have been made in this investigation to determine the mechanism of resistance. It is widely believed that all resistance to purine analogues involves the pyrophosphorylases (Hitchings and Elion, 1963). One can not assume that benomyl is a purine analogue in this phase of the investigation into its mode of action. The evidence presented in this dissertation, when taken collectively, implicates benomyl as interfering with the synthesis of nucleotides, particularly purine nucleotides, so as to deplete the supply available for nucleic acid biosynthesis. The ramifications of these effects are then reflected in the formation of more cell wall material and in the alterations in morphogenesis which have been observed.

BIBLIOGRAPHY

Allen, P. M. and D. Gottlieb. 1970. Mechanism of action of the fungicide thiabendazole, 2-(4'-thiazolyl) benzimidazole. Appl. Microbiol. 20:919-926.

Ashwell, G. 1966. New colorimetric methods of sugar analysis. The phenol-sulfuric acid reaction for carbohydrates, p. 93-94. In E. F. Neufeld and V. Ginsberg (ed.), Methods of enzymology, vol. 8. Academic Press, New York.

Audus, L. J. 1964. The physiology and biochemistry of herbicides. Academic Press, New York.

Banerjee, S. and L. Margulis. 1969. Reversible inhibition of cilia regeneration in Stentor coerulus by isopropyl-n-phenyl carbamate. Nature (London) 224:180-181.

Bartels-Schooley, J. and B. H. MacNeill. 1970. The response of several mutants of Fusarium to benomyl and related fungicides. Phytopathology 60:(Abstr.) 571.

Bartels-Schooley, J. and B. H. MacNeill. 1971. A comparison of the modes of action of three benzimidazoles. Phytopathology 61:816-819.

Bartnicki-Garcia, S. 1968. Cell wall chemistry, morphogenesis and taxonomy of fungi. Annu. Rev. Microbiol. 22: 87-108.

Bent, K. J. and R. H. Moore. 1966. The mode of action of griseofulvin, p. 82-109. In B. A. Newton and P. E. Reynolds (ed.), Soc. Gen. Microbiol., 16th Symp. Cambridge University Press, Cambridge, England.

Betina, V., M. Betinova and M. Kutkova. 1966. Effects of cyanein on growth and morphology of pathogenic fungi. Archiv. fur Mikrobiologie 55:1-16.

Bigger, C. H., M. E. White and H. D. Braymer. 1972. Ultrastructure and invertase secretion of the slime mutant of Neurospora crassa. J. Gen. Microbiol. 71:159-166.

Bollen, G. J. and A. Fuchs. 1970. Specificity of the in vitro and in vivo antifungal activity of benomyl. Neth. J. Plant Pathol. 76:299-312.

Bollen, C. J. and G. Scholten. 1971. Acquired resistance to benomyl and some other systemic fungicides in a strain of Botrytis cinera in cyclamen. Neth. J. Plant Pathol. 77:83-90.

Borisy, G. C. and E. W. Taylor. 1967. The mechanism of action of colchicine. Colchicine binding to sea urchin eggs and the mitotic apparatus. J. Cell Biol. 34:535-548.

Boyle, W. S. 1973. Cytogenic effects of Benlate fungicide on Allium cepa and Secale cereale. J. Heredity 64:49-50.

Brown, H. Dk., A. R. Matzuk, I. R. Ilvues, L. H. Peterson, S. A. Harris, L. H. Sarett, J. R. Edgerton, J. J. Yaktis, W. C. Campbell and A. C. Cuckler. 1961. Antiparasitic drugs. IV. 2-(4'-thiazolyl) benzimidazole, a new anthelminthic. J. Amer. Chem. Soc. 83:1764-1765.

Brusick, D. J. 1972. Induction of cycloheximide resistant mutants in Saccharomyces cerevisiae with N-methyl-N'-nitrosoguanidine and ICR-70. J. Bacteriol. 102:1134-1138.

Bucknall, R. M. 1967. The effects of substituted benzimidazoles on the growth of viruses and the nucleic acid metabolism of host cells. J. Gen. Virol. 1:89-99.

Bucknall, R. M. and S. B. Carter. 1967. A reversible inhibitor of nucleic acid synthesis. Nature (London) 213:1099.

Clemons, G. P. and H. D. Sisler. 1969. Formation of a fungitoxic derivative from Benlate. Phytopathology 59:705-706.

Clemons, G. P. and H. D. Sisler. 1971. Localization of the site of a fungitoxic benomyl derivative. Pestic. Biochem. Physiol. 1:32-43.

Crook, E. M. and I. R. Johnson. 1962. The qualitative analysis of the cell walls of selected species of fungi. Biochem. J. 83:325-331.

Crowdy, S. H. 1970. Crop protection from within the plant. Science J. 6:62-67.

Daines, R. H. and R. D. Snee. 1969. Control of blue mold of apples in storage. Phytopathology 59:792-794.

Dassenoy, B. and J. A. Meyer. 1973. Mutagenic effect of benomyl on Fusarium oxysporum. Mutation Res. 21:119-120.

Davidse, L. C. 1973. Antimitotic activity of methyl benzimidazol-2-yl carbamate (MBC) in Aspergillus nidulans. Pestic. Biochem. Physiol. 3:317-325.

Davidson, E. A. 1966. Analysis of sugars found in mucopolysaccharides. Procedures for free hexosamine, p. 57-58. In E. F. Neufeld and V. Ginsberg (ed.), Methods of enzymology, vol. 8. Academic Press, New York.

Decallone, J. R. and J. A. Meyer. 1972. Effect of benomyl on spores of Fusarium oxysporum. Phytochemistry 11: 2155-2160.

Dee, J. 1966. Genetic analysis of actidione resistant mutants in the Myxomycete, Physarum polycephalum, Schw. Genet. Res. 8:101-110.

Dekker, J. 1969. Acquired resistance to fungicides. World Rev. Pest Control 8:79-85.

Delp, C. A. and H. L. Klopping. 1968. Performance attributes of a new fungicide and mite ovicide candidate. Plant Dis. Reptr. 52:95-99.

deTerra, N. and E. L. Tatum. 1963. A relationship between cell wall structure and colonial growth in Neurospora crassa. Amer. J. Bot. 50:669-677.

Edgington, L. V., K. L. Khew and G. L. Barron. 1971. Fungitoxic spectrum of benzimidazole compounds. Phytopathology 61:42-44.

Egyhezi, E., B. Daneholt, J. E. Edstrom, B. Lambert and U. Ringborg. 1970. Differential inhibitory effect of a substituted benzimidazole on RNA labeling in polytene chromosomes. J. Cell Biol. 47:516-520.

Erwin, E. C. 1969. Methods of determination of the systemic and fungitoxic properties of chemicals applied to plants with emphasis on control of Verticillium wilt with Thiabendazole and Benlate. World Rev. Pest Control 8:6-22.

Frear, D. E. H. 1955. Chemistry of the pesticides. D. Van Nostrand Co., New York.

Friedman, H. C. and D. L. Harris. 1962. Benzimidazole riboside, a possible precursor of vitamin B_{12} in Propionibacterium shermanii. Biochim. Biophys. Res. Comm. 8: 164.

Fuchs, A., A. Homans and F. W. DeVries. 1970. Systemic activity of benomyl [1-(butylcarbamoyl)-2-benzimidazole carbamic acid, methyl ester] against Fusarium wilt of pea and tomato plants. Phytopathol. Z. 69:330-343.

Fuchs, A. G., A. van der Berg and L. C. Davidse. 1972. A comparison of benomyl and thiophanates with respect to some chemical and systemic fungitoxic characteristics Pestic. Biochem. Physiol. 2:191-205.

Godavari, H. R. and E. R. Waygood. 1970. Nicotinamide adenine dinucleotide metabolism in plants. I. Intermediates of biosynthesis in wheat leaves and the effect of benzimidazole. Can. J. Bot. 48:2267-2278.

Gottlieb, D. and K. Kumar. 1970. Effect of benzimidazole on root formation in Phaseolus vulgaris. Acta Agron. (Budapest) 19:372-375.

Gratzner, H. and D. H. Sheehan. 1969. Neurospora mutant exhibiting hyperproduction of amylase and invertase. J. Bacteriol. 97:544-549.

Grove, S. N. and C. E. Bracker. 1970. Protoplasmic organization of hyphal tips among fungi: Vesicles and spitzenkörper. J. Bacteriol. 104:989-1009.

Hammerschlag, R. S. and H. D. Sisler. 1972. Differential action of benomyl and methyl-2-benzimidazolecarbamate (MBC) in Saccharomyces pastorianus. Pestic. Biochem. Physiol. 2:123-131.

Hammerschlag, R. S. and H. D. Sisler. 1973. Benomyl and methyl-2-benzimidazolecarbamate (MBC): Biochemical, cytological and chemical aspects of toxicity to Ustilago maydis and Saccharomyces cerevisiae. Pestic. Biochem. Physiol. 3:42-54.

Hastie, A. C. 1970. Benlate-induced instability of Aspergillus diploids. Nature (London) 226:771.

Hastie, A. C. and S. G. Georgopoulos. 1971. Mutational resistance to fungitoxic benzimidazole derivatives in Aspergillus nidulans. J. Gen. Microbiol. 67:371-373.

Hepler, P. K. and W. T. Jackson. 1968. Reorientation of spindle microtubules by isopropyl N-phenyl carbamate. Proc. Amer. Soc. Plant Physiol. (Abstr.) S-3.

Hine, R. B., D. L. Johnson and C. J. Wenger. 1969. The persistency of two benzimidazole fungicides in soil and their fungistatic activity against Phymatotrichum omnivorum. Phytopathology 59:789-801.

Hitchings, G. H. and G. Elion. 1963. Purine analogues, p. 215-237. In R. M. Hochster and J. H. Quastel (ed.), Metabolic inhibitors, vol. 1. Academic Press, New York.

Hsu, K. S. 1963. The genetic basis of actidione resistance in Neurospora. J. Gen. Microbiol. 32:341-347.

Hunsley, D. and J. H. Burnett. 1970. Ultrastructural architecture of the walls of some hyphal fungi. J. Gen. Microbiol. 62:203-218.

Jones, O. T. G. and W. A. Watson. 1967. Properties of substituted 2-trifluoromethylbenzimidazoles as uncouplers of oxidative phosphorylation. Biochem. J. 102:564-573.

Kaars Sijpesteijn, A. 1970. Biochemical modes of action of agricultural fungicides. World Rev. Pest Control 9:85-93.

Kapoor, M. and E. R. Waygood. 1965a. Metabolism of benzimidazole in wheat. I. Formation of benzimidazole nucleotide. Can. J. Biochem. 43:153-164.

Kapoor, M. and E. R. Waygood. 1965b. Metabolism of benzimidazole in wheat. II. Formation of benzimidazole adenine dinucleotide and its products. Can. J. Biochem. 43:165-171.

Kilgore, W. W. and E. R. White. 1970. Decomposition of the systemic fungicide 1991 (Benlate). Bull. Environ. Contam. Toxicol. 5:67-69.

Kim, W. K. 1970. Effect of excision and benzimidazole treatment on folate content of wheat leaves and wheat leaf chloroplasts. Can. J. Biochem. 48:1091-1094.

Leloir, L. F. and C. E. Cardini. 1957. Characterization of phosphorus compounds by acid lability, p. 840-844. In S. P. Colowick and N. D. Kaplan (ed.), Methods in enzymology, vol. 8. Academic Press, New York.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Lowry, R. J. and A. S. Sussman. 1968. Ultrastructural changes during germination of ascospores in Neurospora tetrasperma. J. Gen. Microbiol. 51:403-409.

Lyda, S. D. and E. Burnett. 1970. Influence of benzimidazole fungicides on Phymatotrichum omnivorum and Phymatotrichum root rot of cotton. Phytopathology 60:726-728.

Mahadevan, P. R. and E. L. Tatum. 1965. Relationship of major constituents of the Neurospora crassa cell wall to wild type and colonial morphology. J. Bacteriol. 90:1073-1081

Mailman, R., E. Hodgson and D. Huisingh. 1971. Effect of thiols in reversing inhibition by methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate on Saccharomyces cerevisiae. Pest. Biochem. Physiol. 1:401-408.

Maxwell, W. A. and G. Brody. 1971. Antifungal activity of selected benzimidazole compounds. Appl. Microbiol. 21:944-945.

McCallan, S. E. A. 1969. History of fungicides, p. 1-37. In D. C. Torgeson (ed.), Fungicides, An advanced treatise. Academic Press, New York.

Meachum, Z. D., Jr., H. J. Colvin and H. D. Braymer. 1971. Chemical and physical studies of Neurospora crassa invertase. Molecular weight, amino acid and carbohydrate composition, and quaternary structure. Biochemistry 10:326-332.

Metzenberg, R. L. 1962. A gene affecting the repression of invertase and trehalase in Neurospora. Arch. Biochem. 96:468-474.

Miller, L. P. 1959. I. Factors influencing the uptake and toxicity of fungicides. Trans. N. Y. Acad. Sci. 21: 442-445.

Miller, N. R. 1972. The nucleotide sequence of "renaturable" leucine transfer ribonucleic acid from Baker's yeast. PhD Thesis, Louisiana State University, Baton Rouge, Louisiana.

Miller, P. M. 1969. Benomyl and thiabendazole suppress root invasion by larvae of Heterodora tabacum. Phytopathology 59:(Abstr.) 1040-1041.

Mishra, D. and E. R. Waygood. 1968. Effect of benzimidazole and kinetin on the nicotinamide nucleotide content of senescing wheat leaves. Can. J. Biochem. 46:167-178.

Nakata, Y. and J. P. Bader. 1969. The uptake of nucleosides by cells in culture: II. Inhibition by 2-mercapto-1-(-4-pyridethyl) benzimidazole. Biochim. Biophys. Acta 190:250-256.

Perkins, D. D. 1959. New markers and multiple point linkage data in Neurospora. Genetics 44:1185-1208.

Perkins, D. D. 1962. Preservation of Neurospora stock cultures with anhydrous silica gel. Can. J. Microbiol. 8:591-594.

Perkins, D., D. Newmeyer, C. W. Taylor and D. C. Bennett. 1969. New markers and map sequences in Neurospora crassa, with a description of mapping by duplication coverage, and of multiple translocation stocks for testing linkage. Genetica 40:247-278.

Person, D. D., D. J. Samborski and F. R. Forsyth. 1957. Effect of benzimidazole on detached wheat leaves. Nature (London) 180:1294-1295.

Peterson, C. A. and L. V. Edgington. 1970. Transport of the systemic fungicide, benomyl, in bean plants. Phytopathology 60:475-478.

Peterson, C. A. and L. V. Edgington. 1971. Transport of benomyl into various plant organs. Phytopathology 61: 91-92.

Reissig, J. L. and J. E. Glasgow. 1971. Mucopolysaccharide which regulates growth in Neurospora. J. Bacteriol. 106:882-889.

Richmond, D. V. and R. J. Pring. 1971. The effect of benomyl on the fine structure of Botrytis fabae. J. Gen. Microbiol. 66:79-94.

Robertson, N. F. and S. R. H. Rizvi. 1968. Some observations on the water relations of the hyphae of Neurospora crassa. Ann. Bot. 32:279-291.

Robinson, H. J., H. F. Phares and O. E. Graessle. 1964. Antimycotic properties of thiabendazole. J. Invest. Dermatol. 42:479-482.

Ryan, F. J., G. W. Beadle and E. L. Tatum. 1943. The tube method of measuring growth rate of Neurospora. Am J. Bot. 30:784-799.

Say, L. G. 1970. Physiological effects of benomyl fungicide on yeast cells. Master's Thesis, Louisiana State University, Baton Rouge, Louisiana.

Schneider, W. C. 1957. Determination of nucleic acids in tissues by pentose analysis, p. 680-684. In S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press, New York.

Schroeder, W. T. and R. Providenti. 1969. Resistance to benomyl in powdery mildew of cucurbits. Plant Dis. Reptr. 53:271-275.

Seiler, P. 1972. The mutagenicity of benzimidazole and benzimidazole derivatives. I. Forward and reverse mutations in Salmonella typhimurium caused by benzimidazole and some of its derivatives. Mutation Res. 15:273-276.

Seiler, P. 1973a. A survey on the mutagenicity of various pesticides. Experientia 29:622-623.

Seiler, P. 1973b. The mutagenicity of benzimidazole and benzimidazole derivatives. II. Incorporation of benzimidazole into the nucleic acids of Escherichia coli. Mutation Res. 17:21-25.

Sels, A. A. 1969. Origin of the increase in adaptability to oxygen of yeast pretreated with benzimidazole. Biochim. Biophys. Res. Comm. 34:740-747.

Shatkin, A. J. and E. L. Tatum. 1959. Electron microscopy of Neurospora mycelia. J. Biophys. Biochem Cytol. 6: 423-426.

Shockman, G. D. 1965. Unbalanced cell-wall synthesis: Autolysis and cell-wall thickening. Bacteriol. Rev. 29:345-358.

Sims, G. M., H. Mee and D. C. Erwin. 1969. Methyl 2-benzimidazolecarbamate, a fungitoxic compound isolated from cotton plants treated with methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (benomyl). Phytopathology 59:1775-1776.

Sisler, H. D. 1969. Effect of fungicides on protein and nucleic synthesis. Annu. Rev. Phytopathology 7:311-330.

Slonimski, P. P. 1954. Sur le mode d'action du benzimidazole sur la levure. C. R. Acad. Sci., Paris 238:1361.

Slonimski, P. P. 1956. Penetration du benzimidazole dans la cellule du levure. Exp. Cell Res. 10:160-167.

Smith, D. G. and R. Marchant. 1969. Unbalanced cell-wall synthesis in chloramphenicol-grown Rhodotorula glutinis. Antonie van Leeuwenhoek 35:113-119.

Solel, Z. 1970. The systemic fungicidal effect of benzimidazole derivatives and thiophanate against Cercospora leaf spot of sugar beet. Phytopathology 60:1186-1190.

Sorenson, S. P. L. 1909. Erganzung zu der Abhandlung: Enzymstudien II. Uber die Messung und die Bedeutung der Wasser stoffionen konzentration bei enzymatischen Prozessen. Biochemische Z. 22:352-256.

Spackman, D. H., W. H. Stein and S. Moore. 1958. Automatic recording apparatus for use in chromatography of amino acids. Anal. Chem. 30:1190-1206.

Stutzenberger, F. J. and J. N. Parle. 1972. Binding of benzimidazole compounds to conidia of Pithomyces chartarum. J. Gen. Microbiol. 73:85-94.

Threlfall, R. J. 1972. Effect of pentachloronitrobenzene (PCNB) and other chemicals on the sensitive and PCNB-resistant strains of Aspergillus nidulans. J. Gen. Microbiol. 71:173-180.

Van Winkle, W. B. 1969. Preliminary observations of the ultrastructure of the slime mutant. Neurospora Newsletter 14:5.

Vogel, H. J. 1956. A convenient growth medium for Neurospora (Medium N). Microbiol. Genet. Bull. 13:42-43.

Wang, D., M. S. H. Hao and E. R. Waygood. 1961. Effect of benzimidazole analogues on stem rust and chlorophyll metabolism. Can. J. Bot. 39:1029-1036.

Weijer, D. L. 1964. Karyokinesis of somatic nuclei of Neurospora crassa: I. The correlation between conidial radiosensitivity and their karyokinetic stage. Can. J. Genet. Cytol. 6:383-392.

Wilkie, D. and B. K. Lee. 1965. Genetic analysis of actidione resistance in Saccharomyces cerevisiae. Genet. Res. 6:130-138.

Westergaard, M. and H. K. Mitchell. 1947. Neurospora. V. A synthetic medium favouring sexual reproduction. Am. J. Bot. 34:573-577.

Woodward, V. W., J. R. deZeeuw and A. M. Srb. 1954. The separation and isolation of particular biochemical mutants of Neurospora by differential germination of conidia, followed by filtration and plating. Proc. Natl. Acad. Sci. U.S.A. 40:192-200.

Woolley, D. W. 1944. Some biological effects produced by benzimidazole and their reversal by purines. J. Biol. Chem. 152:225-232.

Yoshida, Y. 1970. Effect of benzimidazole on the senescence of wheat chloroplasts and their boat shaped transformation. Plant Cell Physiol. 11:435-444.

Zalokar, M. 1961. Electron microscopy of centrifuged hyphae of *Neurospora*. J. Biophys. Biochem. Cytol. 9:609-617.

Table 1. Phenotypic scoring of ascospore isolates from crosses between resistant mutants and alcoy.

Number of mutant		Phenotype: cot+ al	cot+ ylo+	cot+ ylo-	cot- al	cot- ylo+	cot- ylo-	Total; % germination
578	ben^r_s	7	9	0	5	12	0	67
	ben^s	10	10	11	5	0	3	58%
5107	ben^r_s	21	19	0	12	6	0	84
	ben^s	10	1	7	2	0	6	58%
111	ben^r_s	15	18	0	6	15	0	105
	ben^s	14	1	15	10	0	11	89%
119	ben^r_s	11	7	0	17	17	0	102
	ben^s	0	1	8	17	1	13	-
123	ben^r_s	16	6	0	7	7	0	84
	ben^s	16	2	15	11	2	2	-
128	ben^r_s	14	16	0	15	10	0	111
	ben^s	22	0	7	13	4	10	81%
159	ben^r_s	13	11	0	13	9	1	97
	ben^s	15	1	12	14	0	8	77%

Numbers of progeny are given in the body of the table.

APPENDIX

Table 1. Phenotypic scoring of ascospore isolates from crosses between resistant mutants and *alcoy*.

Number of mutant		Phenotype						Total; % germination
		cot^+			cot^-			
		al	ylo^+	ylo^-	al	ylo^+	ylo^-	
578	ben^r	7	9	0	5	12	0	67
	ben^s	10	10	11	5	0	3	58%
5107	ben^r	21	19	0	12	6	0	84
	ben^s	10	1	7	2	0	6	58%
111	ben^r	15	18	0	6	15	0	105
	ben^s	14	1	15	10	0	11	89%
119	ben^r	11	7	0	17	17	0	102
	ben^s	0	1	8	17	1	13	-
123	ben^r	16	6	0	7	7	0	84
	ben^s	16	2	15	11	2	2	-
128	ben^r	14	16	0	15	10	0	111
	ben^s	22	0	7	13	4	10	81%
159	ben^r	13	11	0	13	9	1	97
	ben^s	15	1	12	14	0	8	77%

Numbers of progeny are given in the body of the table.

Table 2. Phenotypic scoring of ascospores isolated from crosses between resistant mutants and strains 1207 and 1208. Numbers of progeny are given in the body of the table.

Number of mutant		Phenotype				Total; % germination
		$ylo^+, tryp^+$	$ylo^+, tryp^-$	$ylo^-, tryp^+$	$ylo^-, tryp^-$	
511	ben^r_s	12	18	3	0	57
	ben^s	0	2	13	9	64%
516	ben^r_s	17	13	1	0	65
	ben^s	1	0	15	18	83%
526	ben^r_s	33	36	1	2	112
	ben^s	0	1	14	25	-
5210	ben^r_s	37	32	0	2	144
	ben^s	6	4	32	32	84%
547	ben^r_s	22	23	1	2	101
	ben^s	0	0	31	22	88%
572	ben^r_s	25	27	0	1	101
	ben^s	0	1	25	22	85%
5810	ben^r_s	18	25	1	0	72
	ben^s	0	1	13	14	-
5102	ben^r_s	35	31	0	4	142
	ben^s	2	3	38	29	-
5107	ben^r_s	22	32	1	1	111
	ben^s	1	2	26	26	90%

Table 2 continued.

Number of mutant		Phenotype				Total; % germination
		$ylo^+, tryp^+$	$ylo^+, tryp^-$	$ylo^-, tryp^+$	$ylo^-, tryp^-$	
5128	ben^r	25	19	1	1	81
	ben^s	1	0	16	18	70%
119	ben^r	32	25	2	1	136
	ben^s	1	5	32	34	95%
123	ben^r	23	22	0	1	107
	ben^s	1	0	36	24	60%
128	ben^r	13	16	1	2	101
	ben^s	0	0	23	46	-
159	ben^r	21	35	1	3	110
	ben^s	4	0	23	23	93%

Table 3. Linkage data for *ben*^{r} relative to *tryp-2* and *ylo-1*.

Zygote genotype: $\frac{+ \quad ben^r \quad +}{tryp\text{-}2 \quad + \quad ylo\text{-}1}$

Number of mutant	Parental combinations	Recombinations			% Recombinations		Total; % germination
		Singles region 1	Singles region 2	Doubles regions 1 and 2	Region 1	Region 2	
511	63	17	0	5	18	4.0	124 75%
519	62	12	0	3	15	2.9	103 86%
526	69	22	0	4	20	3.1	130 82%
5210	73	14	0	6	16	4.8	124 83%
557	52	17	0	3	22	3.3	90 38%
572	69	18	0	4	18	3.2	124 82%
586	59	26	1	1	26	1.8	114 56%
5810	69	13	1	4	15	3.5	113 78%

Table 3 continued.

Number of mutant	Parental combinations	Recombinations			% Recombinations		Total; % germination
		Singles region 1	Singles region 2	Doubles regions 1 and 2	Region 1	Region 2	
5102	60	27	1	2	23	2.4	127 81%
5107	82	19	0	4	14	3.0	132 93%
5128	72	24	2	2	19	3.0	134 56%
111	71	16	1	2	15	2.5	119 84%
123	54	15	1	4	17	3.8	105 66%
128	73	11	0	0	9.6	0	114 89%
159	61	19	0	2	17	1.8	112 93%

Regions are numbered from left to right and numbers of progeny are given in the body of the table.

Table 4. Linkage data for *ben*^{r} relative to *tryp-2* and *chol-2*.

Zygote genotype: $\frac{+ \quad ben^r \quad +}{tryp\text{-}2 \quad + \quad chol\text{-}2}$

Number of mutant	Parental combinations	Recombinations			% Recombinations		Total; % germination
		Singles region 1	Singles region 2	Doubles regions 1 and 2	Region 1	Region 2	
511	63	16	41	6	18	39	124 75%
519	62	8	28	7	15	34	103 86%
526	69	19	35	7	20	32	130 82%
5210	73	15	34	5	16	31	124 83%
557	52	14	17	6	22	26	90 38%
572	69	15	33	7	18	32	124 82%
586	59	14	16	13	24	33	114 56%
5810	69	12	28	5	15	29	113 78%

Table 4 continued.

Number of mutant	Parental combinations	Recombinations			% Recombinations		Total; % germination
		Singles region 1	Singles region 2	Doubles regions 1 and 2	Region 1	Region 2	
5102	60	23	37	6	23	34	127 81%
5107	82	17	27	7	18	26	132 93%
5128	72	18	35	8	18	32	134 56%
111	71	12	30	6	15	30	119 84%
123	54	9	31	10	18	39	105 66%
128	73	5	30	6	9.6	32	114 89%
159	61	15	30	6	19	32	112 93%

Regions are numbered from left to right and numbers of progeny are given in the body of the table.

Table 5. Mapping data for tryp-2, ylo-1, chol-2.

Zygote genotype: $\frac{+ \quad + \quad +}{\text{tryp-2} \quad \text{ylo-1} \quad \text{chol-2}}$

Number of mutant	Parental combinations	Recombinations			% Recombinations		Total; % germination
		Singles region 1	Singles region 2	Doubles regions 1 and 2	Region 1	Region 2	
511	63	12	41	6	15	39	124 75%
519	62	5	27	7	12	33	103 86%
526	69	16	35	8	17	35	130 82%
5210	73	10	34	4	11	31	124 83%
557	52	12	19	5	19	27	90 38%
572	69	11	36	7	15	35	124 82%
586	59	15	27	13	25	35	114 56%
5810	69	8	30	6	12	32	113 78%

Table 5 continued.

Number of mutant	Parental combinations	Recombinations			% Recombinations		Total; % germination
		Singles region 1	Singles region 2	Doubles regions 1 and 2	Region 1	Region 2	
5102	60	28	38	7	21	35	127 81%
5107	82	12	28	7	14	27	132 93%
5128	72	20	35	6	19	31	134 56%
111	71	16	36	1	14	31	119 84%
123	54	9	31	7	15	36	105 66%
128	73	6	30	6	10	32	114 89%
159	61	13	32	6	17	34	112 93%

Regions are numbered from left to right and numbers of progeny are given in the body of the table.

VITA

Mildred Kathleen Borck was born in Tifton, Georgia, on October 31, 1942. She attended public schools in Gainesville, Florida, and graduated from Gainesville High School in 1960. In 1964 she received the Bachelor of Science degree in biology from the University of Florida. She attended the University of California, Riverside, during the 1967-1968 academic year. In the fall of 1969 she entered graduate school at Louisiana State University and is now a candidate for the degree of Doctor of Philosophy in microbiology.

She is married to Jim Springer Borck of Dade City, Florida.

EXAMINATION AND THESIS REPORT

Candidate: Mildred Kathleen Borck

Major Field: Microbiology

Title of Thesis: Studies on the Mode of Action of Benomyl in Neurospora crassa

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

November 29, 1973